VOLUME 26 NUMBER 1

PLANT PHYSIOLOGY

JANUARY, 1951

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PUBLISHED QUARTERLY

BY

THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

AT 131 E. CHESTNUT ST., LANCASTER, PA.

Entered as second-class matter March 16, 1926, at the post office at Lancaster, Pa., under the Act of August 24, 1912.

Accepted for mailing at the special rate of postage provided for in paragraph (d-2), Section 412, Authorized April 3, 1926.

PLANT PHYSIOLOGY

A QUARTERLY JOURNAL
DEVOTED TO ALL PHASES OF PLANT PHYSIOLOGY

Established 1926

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Manuscripts must be typewritten, and must have the approval of the Editorial Board. Papers must conform to the limitations established by the Society at the St. Louis meeting, 1938.

Proofs should be returned with necessary corrections, and reprints ordered when the proof is approved. Reprints can be furnished with or without covers at cost of production.

Correspondence with reference to editorial matters should be addressed to the Editor, Dr. Walter F. Loehwing, Department of Botany, State University of Iowa, Iowa City, Iowa.

Subscription price, $10.00 a year. Single copies, $3.00 post free. Members of the American Society of Plant Physiologists receive the journal in connection with their paid annual membership.

Missing numbers will be supplied if lost in the mail, provided that written notice is received within one month of the date of issue; otherwise they will be furnished at the single copy rate, as long as they are available.

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PLANT PHYSIOLOGY

VOLUME 26 JANUARY, 1951 NUMBER 1

THE INFLUENCE OF LIGHT, TEMPERATURE, AND SOME ENZYME POISONS UPON THE TOTAL ORGANIC ACID CONTENT OF LEAF TISSUE OF *KALANCHOE DAIGREMONTIANA* (HAMET AND PERRIER)

G. Fred Somers[1]

(WITH THREE FIGURES)

Received June 6, 1950

Introduction

Although the phenomenon of the diurnal variation in organic acid content of photosynthetic succulent tissues has long been recognized (**16, 24**), the process is still not understood. Recently considerable interest has been aroused in the problem of organic acid metabolism as a result of the importance assigned to organic acids in the modern hypotheses of intermediary metabolism of carbohydrates, fats, and proteins (**11, 48, 52**). If these acids are as important as is indicated by recent work, particularly with animal tissues (see **20** for a review), the phenomenon of a diurnal variation in organic acid content of succulent tissues becomes one of considerable interest. The main features of this phenomenon of diurnal variation and other aspects of organic acid metabolism in plants have been discussed in various reviews (**5, 6, 39, 48, 56**).

It appears that either temperature or light, or both, are primarily responsible for the observed diurnal fluctuations in the organic acid content of photosynthetic succulent tissues. Some workers have considered light to be the sole or principal factor (**25, 37, 38**). Bennet-Clark concluded that temperature alone is the primary factor and that light acts largely through its effect on the temperature of the irradiated tissues (**5, 6**). Certainly, temperature has a marked effect, and in the dark either acid accumulation or acid breakdown can be induced in leaves by manipulation of the temperature. These changes have been found to be reversible. An accumulation at a low temperature can be reversed by raising the temperature and vice versa (**5, 6, 53, 55**). The effect of temperature is somewhat complicated, however, because it is dependent on other factors such as the previous treatment of the leaves, the oxygen supply, the age of the leaves, etc. (**37,**

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53, 55). It has been found, for example, that sometimes there is an apparent optimum temperature for acid accumulation over short periods of time (**37, 53**). However, over longer periods of time the total amount of acid accumulated appears to be an inverse function of temperature; the lower the temperature, the greater the acid accumulation, at least over the temperature range from about 10° C to about 30° C (**5, 53, 56**).

There is a limited amount of evidence that light may influence the acid content of succulent tissues directly, rather than indirectly through a change in temperature (**54**). It was for the purpose of separating the effects of light and temperature that the first part of the work presented in this paper was undertaken.

The second part of this paper is devoted to studies on the influence of some substances which inhibit the action of various enzymes more or less specifically. Previous work has shown that narcotics influence the changes in the organic acid content of succulent tissues. WARBURG (**50**) and ASTRUC (**3**) found that chloroform vapor markedly or completely inhibited acid breakdown, either in the dark at 35° C or in sunlight. Ethyl ether has a similar influence (**3, 36**). These earlier studies have been confirmed in part by WOLF (**53**) who used low concentration of chloroform (0.0013 to 0.0025 moles per liter of air). These concentrations, he found, did not appear to damage the leaves, yet they produced from 28 to 71% inhibition of acid accumulation. Contrary to the findings of Warburg and Astruc, Wolf found a stimulation of acid breakdown by chloroform in the dark at 37° C. This contradiction still awaits satisfactory explanation.

The majority of the available evidence indicates that oxygen is required for acid accumulation (**3, 19, 36, 37, 50, 53**), hence it would be expected that various oxidizing enzymes which lead either directly or indirectly to the utilization of oxygen are involved in the process. Attempts to study the influence of oxygen upon acid breakdown, especially in the light (**3, 19, 36**) have led to conflicting results. WOLF (**53**) studied the influence of cyanide upon acid accumulation in the dark in leaves of *Sempervivum glaucum* and Bryophyllum spp. A concentration of 0.002 to 0.003 moles per liter of air, which was found not to be "toxic" to the leaves, produced a complete inhibition of acid accumulation. WOLF (**57**) studied also the influence of monoiodoacetic acid and sodium fluoride. These substances (**57**) were found to influence markedly the gaseous exchange of succulent leaves, but their effects upon organic acid changes were not measured.

Materials and methods

The plants used were all *Kalanchoe Daigremontiana* (Hamet and Perrier). (This species is the same as *Bryophyllum Daigremontianum* of previous workers.) This species has the peculiarity of producing small plants in notches along the margins of the leaves while they are still attached to the plant. The plants used in this work were obtained by growing the plantlets from two leaves of one plant in a uniform lot of fertile soil in the greenhouse. The plants were transplanted twice during a period of

six months; the last transplanting occurred about five weeks before the plants were used for experimental purposes.

Usually, before being used for experiments, a number of plants were selected for uniformity in size and were grown for several days to a week or more in glass chambers illuminated for 16 hours each day with tungsten lamps. A layer of water on the top of each chamber prevented most of the heat from the lamps from entering the chambers. The light intensity inside the chambers was reasonably uniform at any particular distance from the lamps, and was about 700 foot-candles at the top of the plants. Lateral buds and small plantlets were removed from the plants periodically. In this manner the growth of the plants was limited to the terminal bud, and the leaves were kept free of plantlets.

Leaves removed for experiments were always placed in a closed container over water as soon as they were removed from the plant. In this way wilting was avoided.

The reagents used were of "Reagent quality" except as follows:

D,L-Malic acid: Eastman practical malic acid was precipitated from anhydrous ether by adding petroleum ether. This was repeated, and the precipitate was dried to constant weight at 70° C in a vacuum oven with a pressure of about 22 cm. of Hg. The material melted at 129° C (uncorrected) and assayed 99.1% pure by titration against NaOH.

Sodium iodoacetate: Eastman mono-iodoacetic acid, M.P., 80.5–82° C, was neutralized with sodium hydroxide. This solution gave a negative test for free iodine.

Sodium azide: This was a portion of some material synthesized by the Chemistry Department at Cornell University. It was obtained through the courtesy of Dr. Paul Marsh.

The method used in assaying for the acids was to grind the tissue in a high speed blender and titrate aliquots of the tissue suspension. Frozen tissue samples were ground directly without thawing, after the addition of a small amount of water. The ground suspension was diluted quantitatively to a convenient volume and the titration was carried out by adjusting an aliquot to pH 7.8 and then titrating with M/20 HNO_3 to pH 2.6 (in some cases to pH 2.5). A quinhydrone electrode was used, and the details of the titration were similar to those used by PUCHER *et al.* (**34**) for the titration of total acids, except that solid quinhydrone was used instead of an acetone solution of quinhydrone.

The influence of some of the variables in this method was tested as follows: Three 20-gram samples of diced leaves were weighed out and placed directly into large Pyrex test tubes. These samples were then stored in an alcohol bath at about minus 25° C. One sample was ground and diluted to 100 ml. after three hours storage at – 25° C. Some aliquots of the resulting suspension were titrated immediately, and the rest of the diluted sample was left in the laboratory desk at room temperature for about 16 hours, when further aliquots were titrated. Another sample was removed from the alcohol bath after 21 hours and similarly ground and titrated. Part of this

suspension was diluted further with an equal volume of water. By this means any appreciable effect of diluting the sample could be detected. The third sample was ground and titrated after being stored for about 95 hours at −25° C. The titration values, corrected for titration of appropriate blanks, are summarized in table I.

From these data it may be concluded that, after correction for the titration of the water used to suspend the tissue, the titration of aliquots containing widely differing amounts of tissue give comparable results. The pH value of the suspended tissue is likewise changed but little by diluting the sample. Hence it is permissible to use varying amounts of tissue. Also, storage in the alcohol bath for as long as 95 hours produces no significant change in the titration value or the pH of the suspended tissue. Similarly, storage of the ground and diluted samples in the dark for a reasonable length of time results in no change. In practice, the samples were titrated

TABLE I

INFLUENCE OF STORAGE AND DILUTION UPON THE TITRATION OF CONTROL LEAF SAMPLES

Sample No.	Temperature of storage	Length of storage	Titration value*	pH of suspension
1	−25° C	3	26.6	5.15
2	−25° C	21	27.8	5.14
2†	−25° C	21	27.5	5.08
3	−25° C	95	27.3	5.21
1††	Room	16	27.0	5.14

*Milliequivalents of acid per 100 g. initial fresh weight of tissue, corrected for the titration of the blank.

†A portion of the suspension of Sample No. 2 diluted with an equal volume of water.

††Suspension of Sample No. 1 stored at room temperature after being ground.

as soon as possible after they were ground although occasionally it was necessary to store them in this condition in the refrigerator overnight.

In most of the experiments, the tissue suspensions were acidified by adding 2 ml. of 3 *N* HCl acid; then they were heated in boiling water before they were titrated. This removed carbon dioxide and gave more consistent results. VICKERY and PUCHER (**46**) reported that oxalic acid could be lost from acidified extracts of plant tissues which were subjected to prolonged vacuum distillation. In the present study, however, it was found that there was no loss of oxalic acid when acidified aqueous solutions of this acid were boiled, and hence it is not considered likely that oxalic acid would be lost from the tissue suspensions. It is important, however, to select an acid for acidifying the tissue samples which, when neutralized with NaOH, gives a salt that interferes as little as possible with the titration. Studies were made using NaCl, Na_2SO_4, and $NaNO_3$. Sodium chloride was found to be satisfactory. In the presence of 0.1 *N* NaCl the titration values with individual organic acids were very nearly the same as in the absence of this

salt, except that the degree of titration of oxalate was reduced slightly, and the degree of titration of tartrate was increased slightly. The titration value of a mixture of oxalate, citrate, dihydrogen phosphate, malate, and tartrate in the presence of 0.1 *N* NaCl was 101.5% that of the similar titration value in the absence of the sodium chloride.

The organic acids likely to be encountered in the titration procedure used, with the exception of oxalic acid, have dissociation constants of about the same order of magnitude. As a result they would all be expected to react similarly in titration. Oxalic acid, however, is a relatively strong acid and only the second acid hydrogen would be expected to be comparable to the acid groups of the other organic acids. The results obtained by previous workers have failed to indicate any changes in oxalic acid comparable to the changes observed in citric and malic acids. Consequently, oxalic acid probably will not introduce serious errors into the titration procedure used. There are other possible sources of error, however, in the titration procedure. One of these might be phosphoric acid and its esters, particularly the hexosephosphates. There is considerable evidence that these esters are present in plant tissues (**4, 15, 21, 40, 41**). In view of the recent work with phosphorylation in plant tissue extracts and intact plant organs (see STAUFFER, **39**) it appears probable that during changes in carbohydrate content there may be changes in the relative amounts of various phosphate esters and their salts. However, most of these esters have dissociation constants of about the same order of magnitude as phosphoric acid (**14, 18**) and hence changes in the relative amounts of these compounds should not introduce a serious error.

It might be suggested that another source of error would arise from changes in various nitrogen fractions. However, WOLF (**53**) showed that these changes were insignificant. In one experiment in the following studies various soluble nitrogen fractions were determined. Ammonia, total amide, and "glutamine" amide fractions were estimated using adaptations of the methods of PUCHER *et al.* (**33**) and VICKERY *et al.* (**47**). Total soluble nitrogen was estimated by an adaptation of the method of UMBREIT and BOND (**43**). The α-amino nitrogen fraction was estimated using a modified Van Slyke manometric apparatus (**44**). No significant changes were found in any of these fractions.

Various titrations of pure acid solutions were made, and the data indicate that since most of the acids in these tissues are either malic, citric acid, or isocitric acid (**5, 7, 8, 13, 29, 30, 54, 56**) the titration procedure used probably gives a good estimate of the total amount of these acids present.

In all of the experiments described below mature, but not old, leaves were used. The margins (about 5 mm.) and the midribs were removed from the leaves with a sharp scalpel. The remaining portions were cut into pieces about 5 mm. square with a thin, sharp razor blade. The pieces were then mixed thoroughly by hand and were stored temporarily in a covered dish over water while the samples for experimental use were weighed rapidly to the nearest 0.01 gram.

Results

Part I. Influence of temperature and light upon changes in acid content

Experiment No. 1.—A number of leaves were removed from plants in the greenhouse at 3:30 to 4:00 p.m., on July 24, which was a hot, sunny day. The leaves were cut into pieces as described above and the mixed pieces were stored temporarily in a covered dish over water while 40-gram samples were weighed out rapidly. Each sample, except for the control samples, was placed in two shallow, paraffined, screen-wire baskets. The baskets were supported over water in large covered culture dishes. Dishes containing one sample each were placed in air chambers in the dark at 31° C, 20° C. One sample was removed from each of the air chambers after 25 and again after 50 hours. Each sample was stored in the alcohol bath at low temperature until it was ground and titrated. The results are summarized in figure 1, which shows that the acid accumulation was greater the lower the temperature. In fact, the data for the highest temperature indicate some acid breakdown during the first 25 hours, followed by an approximately equal acid accumulation during the second 25-hour period. The pH changes are, in general, similar to the acid changes, although there is an indication of disagreement between the two when the changes are considered in detail.

Experiment No. 2.—In this experiment mature leaves from four plants which had received artificial illumination were used. The leaves were picked at the end of the illumination period and at that time the temperature in the chamber was 29° C and such a temperature had prevailed for about eight hours previously. The half-leaves were cut into pieces, and samples were weighed out essentially as in the previous experiment. Three 60-gram samples were placed over water in covered dishes in air chambers at three temperatures in the dark, as above. Two samples were frozen immediately to serve as controls. Two additional 20-gram samples were weighed out, and each was placed in a 250-ml. Pyrex Erlenmeyer flask which was heavily coated with black enamel. To each flask were added 40 ml. of Hoagland and Snyder (17) nutrient solution diluted 10 times. The flasks were stoppered with loose cotton plugs and protected from light by placing a loose aluminum cap over the top of each flask. The samples were then placed in a water bath at 15° C and were shaken mechanically at a speed of about 95 strokes per minute. The water bath was in a room which was dark except for occasional periods when a tungsten lamp was used to permit working in the room.

Samples were removed from the air chambers after 16½ and 39½ hours, respectively. These were frozen as above. Also, after 16½ and 39½ hours, respectively, samples were removed from the flasks in the water bath. Each sample was stored in an alcohol bath and titrated in the same way as the other samples, with a suitable blank being included for the amount of nutrient solution.

The analytical procedure in this and subsequent experiments included

acidification and heating of the tissue suspension before it was titrated. The heated aliquots were allowed to cool and were stoppered tightly until titrated. Just before titration they were diluted to volume and were thoroughly mixed. The free air space in the volumetric flask was small so that only small amounts of carbon dioxide were reintroduced into the sample by these manipulations.

An additional study was made in this experiment. A rather large sample of the initial leaf-pieces was placed over water in a covered dish in the

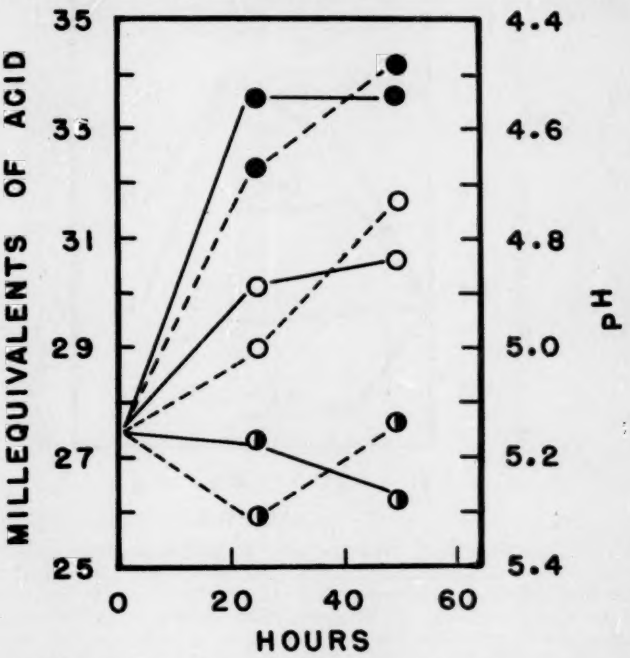

FIG. 1. Experiment No. 1. Titration values (- - - - -) and pH (———) of tissue suspensions prepared from leaf-pieces stored in moist air in the dark at 12°, 21°, and 32° C. The symbols ●, ○, and ◑ represent 12°, 21°, and 32° respectively.

11° air chamber. This sample was removed from the chamber after 40½ hours and three 20-gram subsamples were weighed out. One was frozen for analysis; one was placed in a clear Pyrex Erlenmeyer flask (250 ml.), and the third was placed in a black enameled flask as above. A 40-ml. portion of diluted nutrient solution was added to each sample, and the flasks were placed in the 15° C water bath and shaken mechanically. The clear flask was opposite a 1000-watt, tungsten, spot lamp. This lamp was of the type with a built-in aluminum reflector so that a rather well defined beam of light was produced with comparatively little scattering of the light. The

light was reflected to the flat bottom of the flasks by means of mirrors immersed in the water bath. Between the light source and the water bath was a water filter about 10 cm. deep. The light also passed through about 40 cm. of water in the water bath before it impinged upon the bottoms of the flasks. After 15½ hours the samples were removed from the water bath and treated the same as the other samples from the water bath (above). The results of this experiment are summarized in figure 2. Here again the

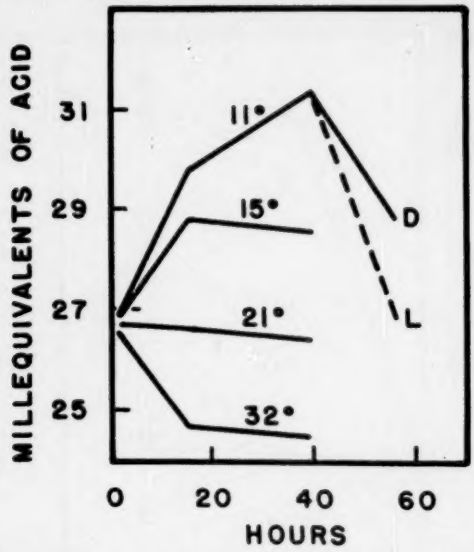

FIG. 2. Experiment No. 2. Titration values of tissue suspensions prepared from leaf-pieces. The samples indicated by 11°, 21°, and 32° were stored in moist air in the dark at these respective temperatures. The sample indicated by 15° was suspended in the dark at this temperature in a nutrient solution. Samples D and L were placed in nutrient solution at 15° C after 39½ hours in moist air, in the dark, at 11°. Sample L was then illuminated, whereas Sample D was not.

organic acid content of the tissue after being in the dark for a period of time is an inverse function of temperature. When a sample which had been at 11° C for 40½ hours was placed at 15° C for an additional 15½ hours the organic acid content decreased. It decreased even more in another comparable sample which was illuminated at 15° C.

EXPERIMENT No. 3.—Mature leaves from a plant grown for 15 days in one of the light chambers were used. The temperature of the light chamber when the leaves were removed was about 33° C and had been at this temperature for about three hours. The leaves were cut into pieces, and the cut pieces were mixed and fourteen 20-gram samples were weighed out. Two of these samples served as controls. They were frozen immediately

in the low-temperature alcohol bath where they were stored until they were analyzed. The remaining samples were treated as follows: Four samples were placed for 12½ hours in each of the dark air chambers, as above, at the temperatures of 31°, 21°, and 11° C. This is the first period shown in figure 3 A, B and C. One sample from each chamber was then analyzed and one sample from each chamber was placed for a second period of 12 hours under each of the following conditions: A, 21° C in dark air chamber (fig. 3 A); B, 21° C in darkened flasks in a water bath, as above (fig. 3 B); and C, in illuminated flasks, as described above, at 21° C in a water bath (fig. 3 C). The results are summarized in figure 3.

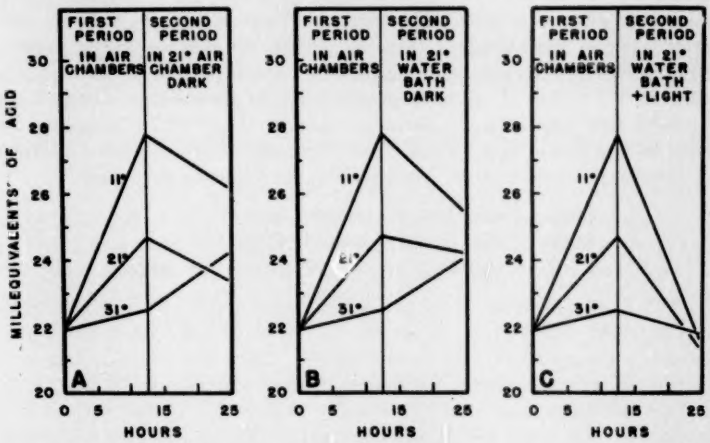

FIG. 3. Experiment No. 3. A comparison of acid changes in pieces of leaf tissue (A) in moist air in the dark, (B) suspended in nutrient solution in the dark, and (C) suspended in nutrient solution and illuminated. All samples were at 21° C during the second period and all of them were in moist air in the dark during the first period. A, B, and C differ only in their treatments during the second period.

During the first 12½ hours the changes in acids were very similar to those in previous experiments with leaf-pieces. The lower the temperature the greater the increase in acids. In fact, the change in acids at 31° was very small. The changes during the second period were influenced by the treatment during the first period. For example, when samples were placed during the second period in nutrient solution in a 21° C water bath (fig. 3 B), the sample from the 21° air chamber showed little change in acid, the sample from the 31° chamber showed an acid increase, and the sample from the 11° chamber showed an acid decrease.

The temperature during the first dark period also influenced rather markedly the acid decrease during the second 12-hour period when the samples were suspended and illuminated in a 21° C water bath (fig. 3 C). The 31° sample showed a slight acid decrease, and the 21° sample a greater

acid decrease, and the 11° sample an even greater acid decrease. As a result the final organic acid content of all of the illuminated samples was about the same. The results of this experiment and the preceding one indicate that similar results are obtained with leaf-pieces whether they are in moist air or whether they are suspended in a nutrient solution and are similar to earlier results obtained with intact plants.

Part II. Influence of enzyme poisons upon changes in acid content

Experiment No. 1. Influence of cyanide and iodoacetate upon acid breakdown.—Mature leaves from a plant which had been in the light chamber for 18 days were used. The leaves were cut into pieces and eight 20-gram samples were weighed from the mixed, cut pieces. These samples were placed in paraffined wire baskets over water in covered dishes in the dark at 11° C for 11 hours to permit acids to accumulate. Two of the samples were then frozen to serve as controls. Each of the remaining six samples was placed in a 250-ml. Pyrex flask and 40 ml. of diluted nutrient solution were added to each. In addition, the following were added:

Flasks 1 and 4: 1 ml. distilled water
Flasks 2 and 5: 1 ml. 0.04 M KCN
Flasks 3 and 6: 1 ml. 0.04 M sodium iodoacetate

The final concentration of potassium cyanide and sodium iodoacetate was M/1000. Flasks 1, 2, and 3 were clear flasks and flasks 4, 5, and 6 were painted on the outside with black enamel. After the solutions had been introduced into the intercellular spaces of the leaf pieces by vacuum infiltration the flasks were placed in a water bath at 25° C and were shaken mechanically at about 100 strokes per minute. Each clear flask was illuminated from below with a 1000-watt lamp as described above. All of the flasks were stoppered with rubber stoppers, and the tops of the enameled flasks were further protected against light by aluminum caps. All of the samples were removed from the water bath after 12 hours and stored immediately in an alcohol bath at about minus 25° C until analyzed. The temperature of the solution in one of the enameled flasks was measured just before it was taken from the water bath and found to be 24.9° C. All of the samples were ground in the solutions used to suspend them and the controls were ground in a similar aliquot of diluted nutrient solution. The ground samples were acidified, heated, and titrated as previously. The results are summarized in table II.

Experiment No. 2.—The details of this experiment were very similar to those of the previous experiment. In this case the acid titration value was determined at the beginning of the experiment as well as after the first period in the dark at 11° C and after the second period at 25° C. The light intensity at the bottom of the flasks was about 2600 to 2800 fc. The results are summarized in table III. In both of these experiments the acid decrease, both due to temperature and to the combined effect of light and

TABLE II

EXPERIMENT NO. 1. THE INFLUENCE OF M/1000 CYANIDE AND IODOACETATE UPON ACID BREAKDOWN IN THE LIGHT AND DARK

Treatment	Titration*	Change in titration	Per cent. inhibition
Control	28.1	...	...
Dark Samples: from 25° water bath			
Water added	24.4	−3.7	...
Cyanide added.....	27.2	−0.9	76
Iodoacetate added....	26.2	−1.9	49
Illuminated Samples: from 25° water bath			
Water added	22.2	−5.9	...
Cyanide added.....	27.3	−0.8	87
Iodoacetate added....	26.5	−1.6	73

*Milliequivalents of acid per 100 grams fresh weight.

temperature, was inhibited strongly by both M/1000 cyanide and M/1000 iodoacetate.

EXPERIMENT NO. 3. INFLUENCE OF FLUORIDE, AZIDE, AND PYROPHOSPHATE UPON ACID BREAKDOWN IN THE LIGHT.—This experiment was carried out essentially as described above, except that the first series of samples was removed from the 11° incubator after 16 hours and the second series of samples was removed after 28 hours. Samples from both series were infiltrated with M/1000 solutions of sodium pyrophosphate, sodium fluoride, or sodium azide, and were then illuminated in the water bath at 25° C. The shaker speed was about 120 strokes per minute. The light intensity at the bottom of the flasks was about 3200 fc. After 12 hours, the samples were removed from the water bath and frozen until analyzed. The results are summarized in table IV.

TABLE III

EXPERIMENT NO. 2. THE INFLUENCE OF M/1000 CYANIDE AND IODOACETATE UPON ACID BREAKDOWN IN THE LIGHT AND DARK

Treatment	Titration*	Change in titration	Per cent. inhibition
Initial	25.2	...	...
First Period: in 11° chamber in the dark			
	28.0	+2.8	...
Second Period: in 25° water bath, in the dark			
Water added	24.8	−3.2	...
Cyanide added.....	27.0	−1.0	69
Iodoacetate added....	27.3	−0.7	78
Second Period: in 25° water bath, illuminated			
Water added	22.0	−6.0	...
Cyanide added.....	26.0	−2.0	67
Iodoacetate added....	26.2	−1.8	70

*Milliequivalents of acid per 100 grams fresh weight.

The most striking feature of these results is the fact that M/1000 sodium azide gave almost identically the same degree of inhibition as was observed with M/1000 cyanide. The mean value with cyanide (light and dark) was 75% inhibition; with azide the corresponding value was 77%. On the other hand M/1000 fluoride shows only a slight inhibition, and M/1000 pyrophosphate shows no inhibition of acid breakdown in the light. It is possible that the pyrophosphate did not enter the cells (51). The inhibition with fluoride is of interest since the concentration used was only M/1000. Usually somewhat more concentrated solutions are used.

TABLE IV

THE INFLUENCE OF M/1000 FLUORIDE, AZIDE, AND PYROPHOSPHATE UPON ACID BREAKDOWN IN THE LIGHT IN THE WATER BATH AT 25° C.

Treatment	Titration ††	Change in titration	Per cent. inhibition
First Series:			
Control*	31.8	...	...
Water added	22.7	− 9.1	...
Azide added	31.6	− 0.2	78
Fluoride added	24.5	− 7.3	20
Pyrophosphate added	23.2	− 8.6	6
Second Series:			
Control†	32.0	+ 0.2	...
Water added	22.2	− 9.8	...
Azide added	29.6	− 2.4	76
Fluoride added	24.1	− 7.9	19
Pyrophosphate added	21.7	−10.3	− 5

*The control in this case is the sample which was frozen immediately after the first 16 hours in the air chamber at 11° C. It should be noted that the titration value of the initial material was not determined so that no statement can be made concerning acid changes during the dark period at 11° C.

†Frozen immediately after 28 hours in the air chamber at 11° C. The recorded change in titration for this sample is the change during the period from 16 to 28 hours.

††Milliequivalents of acid per 100 grams fresh weight.

INFLUENCE OF ENZYME POISONS UPON ACID ACCUMULATION.—This experiment was carried out in the dark essentially as in the experiments described above, except that the differential treatments were applied to the leaf-pieces from the start so that the effect of the various enzyme inhibitors upon acid accumulation could be observed. The plants for use in this experiment were removed from the illuminated chamber and were placed in the greenhouse for about 30 hours before the leaves were removed. This was done to lower the initial acid content as much as possible. Mature leaves were used as above and they were removed from the plant late in the afternoon following a bright, sunny day. The temperature in the greenhouse had been between 36° and 40° C for about five hours prior to the removal of the leaves.

The leaves were cut into pieces, mixed, and weighed into samples of 20 grams each. Two samples were frozen immediately and the remainder were

suspended in diluted nutrient solution. Two of these samples were infiltrated with nutrient solution only, and one of each of the remaining samples was infiltrated with a M/1000 solution of an enzyme inhibitor in nutrient solution. After vacuum infiltration, the samples were shaken in the dark in a water bath at 10.5° C for 15 hours. The samples were then analyzed as in previous experiments. The results are summarized in table V. These data indicate a strong inhibition of acid accumulation by both M/1000 iodoacetate and M/1000 azide.

TABLE V

THE INFLUENCE OF M/1000 AZIDE AND IODOACETATE UPON ACID ACCUMULATION IN THE DARK AT 10.5° C.

Sample	Titration*	Increase in titration	Per cent. inhibition
Initial	23.5	...	...
Samples from water bath:			
Water added.....	27.6	4.1	...
Azide added	24.0	0.5	88**
Iodoacetate added ...	23.4	− 0.1	102

* Milliequivalents of acid per 100 grams fresh weight.

** The degree of inhibition is calculated in terms of the increase in titration of the sample to which only water was added.

Discussion

While the results of these experiments are presented merely in terms of titration values it is probably safe to assume that they reflect the changes which occur in the organic acid content of the tissues involved. Earlier work has characterized the acids which occur in leaf tissues similar to these. The acids found are principally malic, citric, and isocitric acids, and of these, citric and malic acids seem to be chiefly responsible for the variations in organic acid content in response to various factors (**32**). It would, of course, be desirable to have detailed analyses of all such tissues in all experiments. However, this consumes a rather large amount of time and it is probable that measures of the total amount of organic acids present are of significance when something is known about the acids likely to be influenced. To the extent that this assumption is valid the above results can be interpreted as a measure of the influence of the various factors upon the changes of the organic acid content of these tissues.

The results of these experiments show rather clearly that both illumination and an increase in temperature can cause acid breakdown in leaf-pieces. The responses observed in these leaf-pieces are very similar to those reported earlier for intact leaves and intact plants. The use of leaf-pieces suspended in an aqueous medium has made it possible to separate clearly the effect of temperature from that of illumination. Both factors influence the organic acid changes in these tissues and when the two factors are acting simultaneously the changes are greater than with either factor alone.

Furthermore the effects of light and temperature are determined by the previous history of the material. This is shown particularly by the differences in response following the storage of leaf-pieces at various temperatures prior to the introduction of differential light and temperature treatments.

With respect to the influence of the various enzyme poisons upon the changes in organic acid content of these tissues, it is not possible to give a complete and satisfactory interpretation of the observed changes because so little is known of the intermediate reactions involved in organic acid metabolism in plant tissues. It is probable that a modified Krebs tricarboxylic acid cycle occurs in such tissues. The evidence in support of this has been summarized recently by STAUFFER (**39**). More recent experimental support has been supplied by LATIES (**22, 23**), PUCHER and VICKERY (**31**), and VICKERY and ABRAHAMS (**45**). If such a cycle occurs in succulent tissues, pyruvic acid might be the precursor of those organic acids which change so greatly in concentration in response to changes in environmental factors. Pyruvic acid, or other similar compounds, has previously been suggested as a precursor of these organic acids (**20**). By analogy to the intermediary carbohydrate metabolism for yeast and muscle, the formation of pyruvic acid from sugars would be expected to be inhibited by iodoacetate since this substance inhibits triosephosphate dehydrogenase (**1**). If such is the case, the accumulation of organic acids such as citric, malic, and isocitric acids would be inhibited by this poison. The inhibition of organic acid accumulation by iodoacetate, which was observed above, is consistent with such a hypothesis.

Iodoacetic acid may influence acid breakdown by its inhibitory action upon isocitric acid dehydrogenase (**2**), if it is assumed that some sort of tricarboxylic acid cycle occurs in succulent plant tissues. This same action of iodoacetate may account both for the inhibition of acid breakdown and the inhibition of acid accumulation. In any case, the action of iodoacetate indicates that sulfhydryl-containing enzymes are responsible for the changes in organic acid content of these tissues.

It has been suggested that iron- and copper-containing enzymes serve as terminal oxidases in plant respiration (**9, 26, 49**). Such enzymes presumably would transfer electrons which arise in the tricarboxylic acid cycle to oxygen. This action may be intermediated by one or more intermediate hydrogen transfer mechanisms. As mentioned above, it has been observed that oxygen is required for organic acid accumulation and according to the hypothesis that the amounts of the different acids present is the result of reactions in the tricarboxylic acid cycle it would also be expected that oxygen would be required for the breakdown of these acids. Hence, it is to be expected that both azide and cyanide would inhibit both acid accumulation and acid breakdown as was observed.

ULRICH (**42**) found that both the increase and the decrease in the organic acid content of barley roots could be influenced by various ions, *e.g.*, when cations were accumulated from the nutrient solution in excess of anions,

organic acids were formed. Similar responses have been observed by other workers with other plant materials (**10, 12, 27, 28, 35**). It is unlikely that the organic acid changes described above are the result solely of such a differential in ion accumulation from the nutrient solution since the same anions, *e.g.*, azide and iodoacetate, can inhibit both the formation and breakdown of organic acids.

Summary

1. By use of a simple titration technique, changes in the organic acid content of leaf-pieces of *Kalanchoe Daigremontiana* (Hamet and Perrier) were studied. It was demonstrated that both illumination and raising the temperature cause an acid breakdown. The effects of these two factors are conditioned by the previous history of the leaf tissue.

2. Azide, cyanide, and iodoacetate when infiltrated into the tissue at a concentration of M/1000 were found to inhibit strongly the breakdown of organic acids, both in the light and in the dark. M/1000 fluoride produced some inhibition, but pyrophosphate had no effect.

3. Azide and iodoacetate, at a concentration of M/1000, inhibited strongly acid accumulation in the dark.

The writer gratefully acknowledges the advice and constructive criticism offered by Professors O. F. Curtis and Lewis Knudson while this work was in progress.

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THE BIOELECTRIC POTENTIAL OF SEEDS AS A FUNCTION OF GROWTH AND OF X-RAY DOSAGE

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(with six figures)

Received June 26, 1950

Introduction

Radiation in its passage through matter causes ejection of electrons from atoms on which it impinges. The ejection of electrons may result in both chemical and physical changes in the constituents of cells. These changes are considered to be primarily responsible for the biological effects of radiation.

When a cell is subjected to x-radiation, small islands of chemical change are produced in the region where energy quanta are absorbed. Because radiation penetrates more or less uniformly all parts of small living systems, these islands are produced probably in all the cell parts (membranes, cytoplasm, nuclei and chromosomes). Thus in an organic system that is complex to start with, many different kinds of molecules are destroyed and likewise many new ones come into existence in a chaotic fashion. These changes produce disorganization in either functional or control activities of the cells, or in both. Experimental observations (**4**) show that generally functional activity is not easily affected by radiation because of the continuous supply of new material through nutrition. Such processes as respiration, metabolism, and enzymatic activity were found to be little influenced by radiation. Dale (**4**) has shown that only very large doses reduce the activity of enzymes. This reduction can have little importance in tissue unless the sources of enzymes are inactivated.

The exact origin of bioelectric potentials is still open to question although a great deal of work has been done to determine the cause and the seat of the electrical asymmetry responsible for the e.m.f. (**5, 6**). However, it is recognized that in all biological systems there are a great number of phase boundaries with resulting differences of potential. There undoubtedly are many membrane potentials created by a difference of concentration of the electrolyte on the two sides of each membrane. The islands of ionization produced by the passage of x-rays through biological materials together with the chemical changes caused thereby are bound to affect the bioelectric potentials under any plausible theory of the origin of these potentials.

The object of these experiments is to measure the bioelectric potentials of seeds as a function of growth and of x-ray dosage.

Apparatus and procedure

The electrode system used for measuring the bioelectric potentials was patterned after that of Burr and associates (**7**). Contact with the speci-

men was made by means of brushes through which flowed 0.1 normal NaCl solution from test tubes in which were immersed Ag and AgCl electrodes.

The silver chloride electrode was prepared by coating a small coil of silver wire electrolytically with silver chloride, a current of 1 ma. being passed through a 10% solution of NaCl for four hours with the silver wire serving as anode and platinum wire as the cathode. Several sets of electrodes were prepared and those which seemed to be the most uniformly coated were used in the experiment.

A somewhat simplified electrode system was used with good results in later experiments. A glass tube about eight inches long and about $\frac{3}{8}$ inch in diameter was bent in the form of an L, and the lower part drawn out and stoppered with about $\frac{1}{8}$ inch of felt. The metal electrode was introduced into the tube until it touched the felt. The tube was filled with salt solution and sealed at the upper end with wax. The moist felt makes fairly good electrical contact. Care was taken to use two tubes which allow the liquid to pass at the same rate.

In one set of experiments the electrodes were connected by means of shielded wires to a sensitive galvanometer. A potentiometer in series with the galvanometer was used to calibrate the system and to balance out the contact potential. The sensitivity of the system was about 30 microvolts division and was linear over the total range used. The resistance of the path was of the order of two megohms; thus the current drain from the tissue was relatively small. After each measurement the seed was turned through an angle of 180° and a similar measurement taken in the reverse direction. The fact that these two measurements were always in agreement was taken as evidence that the draining of the specimen was small and the circuit was functioning satisfactorily.

In later experiments the electrodes were connected to an electrometer circuit (**3**) using an FP54 tube with grid resistances ranging from 5×10^7 to 10^{11} ohms. The results were essentially similar.

The x-ray tube was operated at about 60,000 volts. The radiation was filtered through $\frac{1}{2}$ mm. of aluminum. At the location of the seeds the dosage was about 400 r/min.

The seeds were grown in a tray on absorbent cotton, moistened with ordinary tap water. For the measurement of the potential, a seed was placed on a small insulated stand mounted on a revolving base. The brush contacts, which were mounted on adjustable, flexible lever arms, were, in the case of corn, lightly touched to the germinal and micropylar end, *i.e.*, along the longitudinal axis. The galvanometer deflection was noted, then the seed was rotated 180° by turning the base and the measurement of the seed repeated with the reversal of polarity of the e.m.f. to the galvanometer. In the case of corn a large initial deflection was designated the primary potential of the seed and the secondary steady state deflection was designated the steady potential. Nelson and Burr (**7**) also report a primary and steady state potential of corn seeds. The mean value of these deflec-

tions on the two sides of the zero position was used for the calculation of the bioelectric potential of the individual seed. After a number of preliminary experiments, it was finally decided that a group of 30 specimens was sufficient to give a fair measure of the bioelectric potential at any period of growth of the seeds. This same procedure was followed for the determination of the effects of radiation on the potential and for other studies. The temperature was kept reasonably constant during experimentation. The effects of polarization of the electrodes were negligible and the balancing out of the contact potential was checked at frequent intervals and properly adjusted when necessary.

Results

Bioelectric potential of corn seeds

The corn seeds used for this study were of an inbred variety developed at the University of Florida Agricultural Experiment Station. These seeds are incapable of high yields and were chosen for the particular study of potential versus growth because it was believed that the range of variations of potentials of different specimens for this specific group of seeds would be much lower than the range of variations of potentials of hybrid seeds. This was found to be true as is shown in table I. In most cases, as found by

TABLE I

COMPARISON OF BIOELECTRIC POTENTIALS OF LOW YIELD AND HIGH YIELD TYPES OF CORN SEED

(MEASUREMENTS WERE MADE AFTER 24 HOURS GROWTH. EACH GROUP CONSISTS OF 30 SEEDS.)

Seed	Average steady potential	Range	Average primary potential	Range
	mv.	*mv.*	*mv.*	*mv.*
Inbred (Low yield)	1.65	0.2– 5.8	3.70	0.3–12.9
Hybrid, Oklahoma Silver Mine (High yield).....	3.68	0.6–30.0	4.90	1.0–21.0

Nelson and Burr (7), the germinal end of the seed was positive with respect to the micropylar end.

In figure 1, the average steady and the average primary bioelectric potential in millivolts is plotted against the number of hours of growth of the various groups of seeds. It is evident that the increase of the potential is very gradual during the first 30 hours in solution. After 30 hours growth the increase in potential is more pronounced.

It was decided to use a vigorous hybrid type seed for the determination of the effects of x-radiation on the bioelectric potential of corn seeds. Since

the potential of hybrid seeds is higher than that of the inbred variety, it was reasonable to expect a greater variation of the potential with x-ray dose. Tests made on two hybrid varieties, White Dent and Oklahoma Silver Mine, showed that Oklahoma Silver Mine gave higher potentials. It was therefore chosen for study.

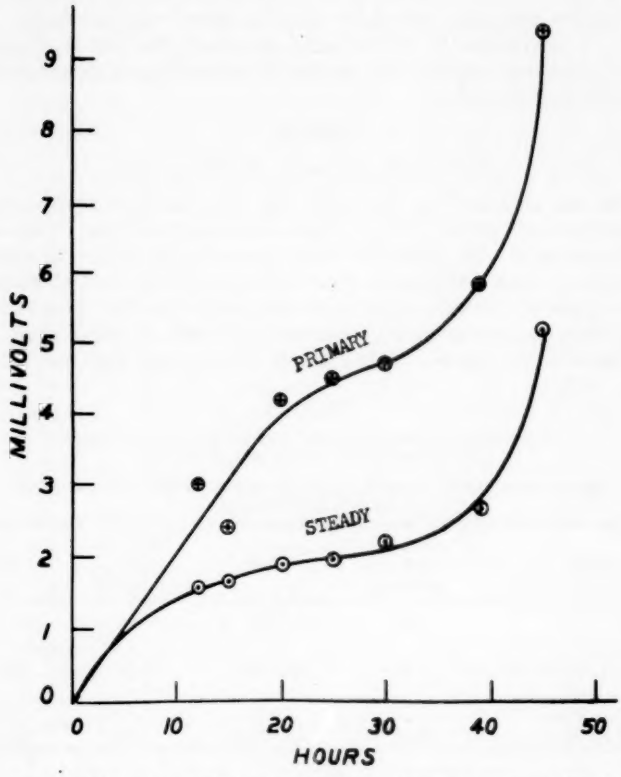

FIG. 1. Bioelectric potential of corn seeds as a function of time of growth.

Comparison between the above data and those observed for inbred seeds (low yielding) shows that the average steady potential of White Dent corn seed for the same time of growth is about twice that of the inbred variety, and the potential of Oklahoma Silver Mine about three to four times that of the inbred variety. These results confirm the conclusions of NELSON and BURR (7) that a higher bioelectric potential indicates a type of seed with a greater yield.

Eight groups of 30 seeds each were planted and six of these groups were

treated with different doses of x-rays ranging from ½ minute to 10 minutes (*i.e.*, 200 r to 4000 r), the other two groups serving as control. The seeds were irradiated 21 hours after immersion in nutrient solution. The average steady and primary potentials of all seeds were measured at 24 hours of growth (*i.e.*, three hours after irradiation) and again at 48 hours.

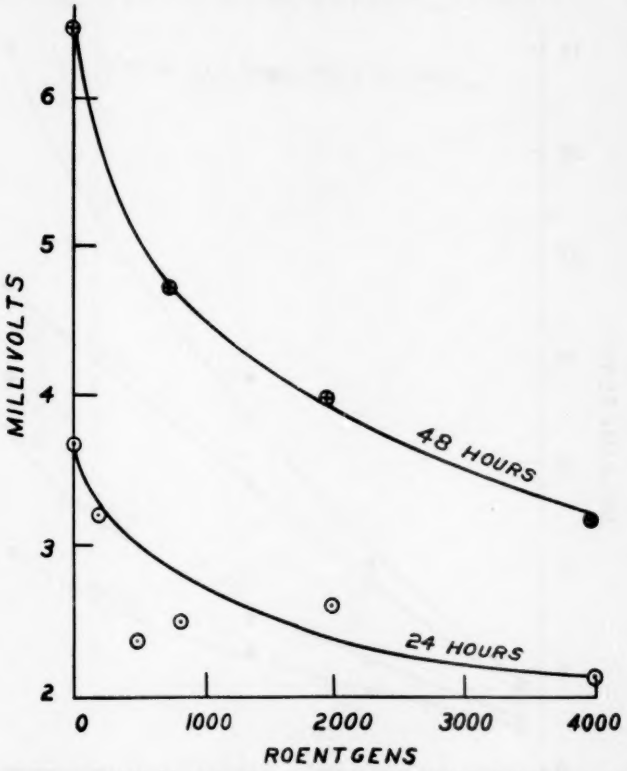

FIG. 2. Bioelectric potential of corn seeds at 24 and 48 hours' growth as a function of dosage.

Figure 2 shows the variation of the average steady potential with x-ray dose at 24 and 48 hours growth. The decrease of the potential of the seeds from the normal control group is quite evident. However, even for a dose of 4000 r the bioelectric potential of the seeds is still about one-half that of the mean control which indicates that the dosage given only served to lower the vitality of the seeds but not to kill them. The fact that at 48 hours the potential is higher than at 24 hours is also evidence that the largest dose

was not lethal. This indicates that the growth rate for the irradiated groups approaches the growth rate of the control. Thus, on the basis of LEA's work (**4**), one would assume that in time the irradiated seeds would have recovered completely. It was not known whether the injury was of a permanent nature or not, since the seeds were not grown to maturity.

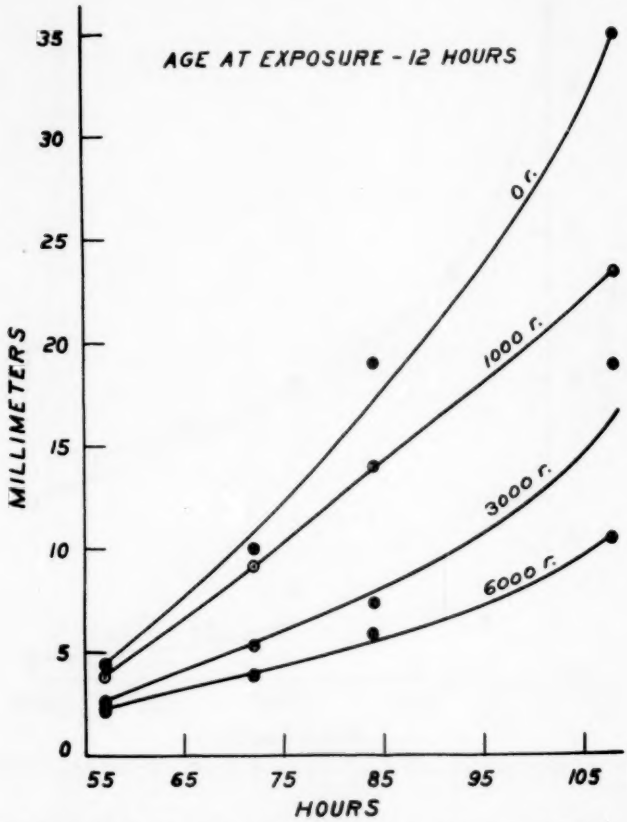

FIG. 3. Length of coleoptile of wheat seeds subjected to various radiation doses as a function of time of growth.

It is noted that a dose of 4000 r seems to lower the bioelectric potential slightly more than does a dose of 800 r. The fact that the curve approximates an exponentially decreasing function is in accordance with the radiation studies of Lea.

At the time of measurement of the 48-hour potentials of the irradiated seeds the sprouts of the various groups of seeds were counted. The length

of the sprouts was not measured. It was noted that the 200 r dose did not affect the sprouting, but as the dose increased the number of sprouts became less.

Two different groups of 30 seeds, small and large, were selected with regard to relative size from a large batch, then weighed and grown for a period of about 47 hours under standard conditions as outlined above. The

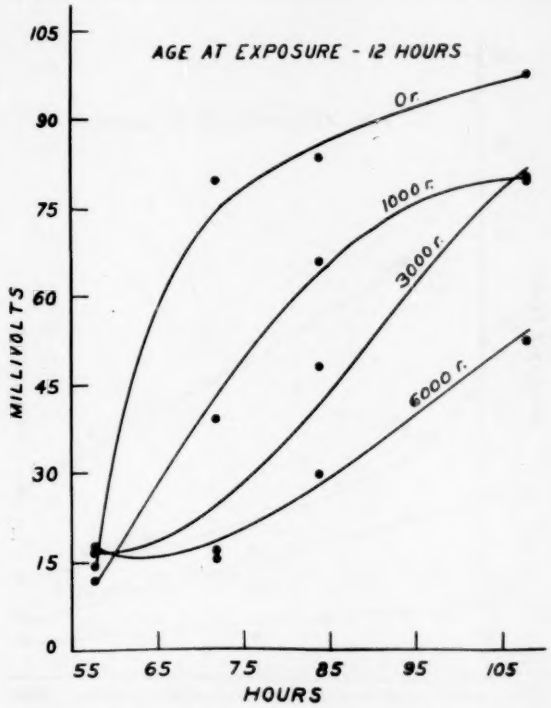

FIG. 4. Bioelectric potential of wheat seeds subjected to various radiation doses as a function of time of growth.

seeds chosen for this study were of the inbred type used for the growth study presented earlier. The potentials of seeds were measured along the longitudinal axis. No significant change in the bioelectric potential with weight of the seeds was observed.

BIOELECTRIC POTENTIAL OF WHEAT SEEDLINGS

The work with wheat was particularly interesting because wheat seedlings at a very early stage of their growth provide easily measurable coleoptiles, the length of which may be taken as an index of the vigor of the

plant (1). The difference of potential was measured between the tip of the coleoptile and its base. Since the primary and steady potentials were very nearly the same no attempt was made to distinguish between them.

Four groups of seeds have been subjected to various doses of x-rays 12 hours after immersion in the nutrient solution, and the length of the coleoptile, as well as the magnitude of the potential of each seedling, were measured. The series of curves in figure 3 shows the variation with time

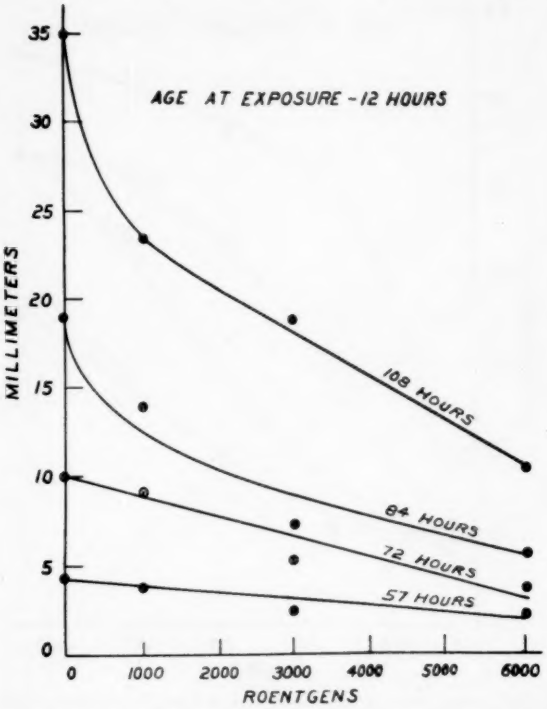

FIG. 5. Length of coleoptile of wheat seeds at various stages of growth as a function of dosage.

of growth of the average length of coleoptile for each group for various doses. The curves show that the inhibition of growth of the coleoptile by irradiation takes place very soon after the dose is administered though the effects at first are not very great. The diminution in the length of the coleoptile with dose becomes more pronounced as time goes on; however, even the largest dose (6000 r units) administered under these conditions (after 12 hours in solution) is not lethal since the coleoptile still continues to grow.

This is even more clearly demonstrated in figure 4, showing the variation with time of growth of the average potential of each group subjected to different doses. The heavy doses of radiation seem to inhibit cell activity greatly only at the beginning, but after 72 hours or so the seed seems to overcome, partly at least, the paralyzing effects of the rays and cell activity is resumed with great vigor. Figures 5 and 6 show the effect of radiation on the length of coleoptile and on the potential of the seed at various stages

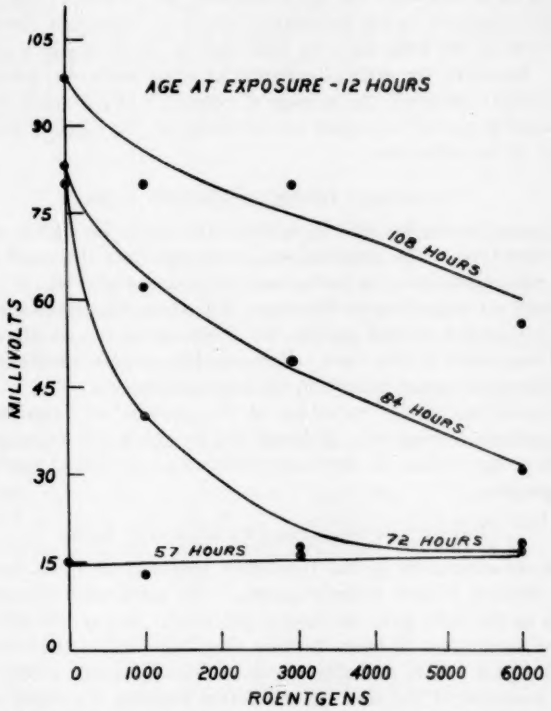

FIG. 6. Bioelectric potential of wheat seeds at various stages of growth as a function of dosage.

of its growth. At 50 hours the effects are not significant. The effects become more pronounced as the seed develops, with respect to both the length of the coleoptile and the magnitude of the potential. The reliability of potentials as a measure of radiation injury is discussed elsewhere (**1**).

It is interesting to note that while the range of variation of the potentials of wheat seeds was similar to that of corn seeds, with a 20-fold variation within a given group not being uncommon, the range of variation of

the lengths of coleoptiles was much smaller. Neglecting those that failed to develop at all an eightfold variation within a given group was quite rare. This is to be expected since the length of the coleoptile is the result of the activity of the seed over a considerable period, so that individual variations are greatly reduced. On the other hand, the potentials indicate the activity of the seed at the time of measurement or at a time immediately preceding it, with consequent great variations.

This is consistent with the other observed fact; namely, that seedlings with large coleoptiles do not necessarily give large potentials, since the cell activity even of the most vigorous seed may be small during a given time interval. However, the average potential of a few seeds over a long period of time, or the equivalent, the average of potentials of a number of seeds at a given time is probably as good an indication of the vigor of the seed as the length of the coleoptile.

Bioelectric potentials of bean seeds

The beans chosen for this study were the Lady Bountiful, a hybrid, high-yielding type. The potential was measured from the point at which the seed was attached to the pod across to the opposite side.

Although the magnitude of the potential is about four times that of corn seeds at any period of their growth, the similarity of the growth curves of corn and bean seeds is very close. Because of the marked similarity, it was not considered necessary to include the bean-seed curves.

Determinations of the variations of the potential of bean seeds with weight were also carried out. Although the weight of the larger group was twice that of the smaller, no significant difference in potential was noted for the two groups.

Bioelectric potentials of lima bean seeds

A few measurements of the bioelectric potential of Lima beans were made at different periods of their growth. The seeds were measured from tip to tip as this axis gave the largest potential. It was difficult to work with these seeds after 16 hours because they burst out of their outer shell and crack apart. This probably forms an injury current which obscures the true potential of the seed. Even before bursting the seeds are quite porous, and the physiological salt solution from the contact brushes quickly penetrates into the internal part of the seed and over the surface with a resultant shorting-out of the potential.

The average steady potential of the seeds just before bursting was about 3.5 mv with a range of 0.5–10 mv.

Summary

As a result of this study of the bioelectric potentials of certain seeds, the following conclusions can be made:

1. The high-yielding seeds have higher bioelectric potentials than those of low-yielding varieties.

2. The bioelectric potential of seeds is zero at dormancy and rises slowly during the first few hours in a humid environment. The potential rises much more rapidly as soon as coleoptile elongation begins.

3. The variation of bioelectric potential with x-ray dosage follows closely the variation of the length of coleoptile with dosage. X-rays, in general, decrease the bioelectric potential of plant seeds and lower their vitality.

4. The bioelectric potential is not a function of the weight of the seeds.

Our thanks are due to Mrs. June Jones for her help in obtaining some of the data. A part of the data presented in this paper was obtained under the auspices of the Atomic Energy Commission.

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EFFECTS OF RESPIRATION INHIBITORS ON ACCUMULATION OF RADIOACTIVE PHOSPHORUS BY ROOTS OF LOBLOLLY PINE

PAUL J. KRAMER

Received August 5, 1950

The close correlation between mineral nutrient accumulation and metabolic activity of roots has become well established during the past two decades (**3**). This correlation is based largely on observations of the effects of aeration, temperature, and carbohydrate supply of the roots on accumulation of nutrients. It would be expected that substances inhibiting root respiration would inhibit the accumulation of minerals. In one of the few studies involving the use of respiration inhibitors MACHLIS (**7**) reported that cyanide and azide inhibited two-thirds of the respiration of barley roots and completely inhibited accumulation of bromide ion, and there was indirect evidence that potassium accumulation was inhibited. Iodoacetate and malonic acid also inhibited both respiration and salt accumulation of barley roots. LATIES (**6**) likewise reported that iodoacetate, malonic acid, and fluoride inhibit respiration of barley roots, but he did not study their effects on salt accumulation.

No studies of this sort have been made on roots of woody species. A series of experiments was therefore performed to find whether or not respiration inhibitors reduce mineral accumulation in loblolly pine (*Pinus taeda* L.) roots as they do in barley roots. An attempt was also made to learn whether or not mycorrhizal roots react in the same manner as non-mycorrhizal roots. Since mycorrhizae represent a complex structure, consisting of both root tissue and fungal hyphae, it seemed possible that they might differ physiologically from those roots which have no fungus associated with them.

Experiments with respiration inhibitors

Roots were obtained from potted loblolly pine seedlings growing out of doors and from seedlings growing in a clearing in the forest. The root tips and segments bearing mycorrhizae were freed of foreign matter by gently brushing with a camel's hair brush in a shallow tray of distilled water. A total of 48 segments, each at least 2 cm. in length and bearing a healthy, growing tip, were selected and separated into four groups for the comparison of the various inhibitors. Since it was impossible to find 48 root tips of the same diameter, the tips were first divided into 12 groups, each containing four roots of approximately the same dimensions. One root from each group was then used in each treatment so the various sizes of roots were uniformly distributed among the four treatments. Roots bearing mycorrhizal branches were cut into segments 1 cm. in length and selected

for uniformity in size and number of mycorrhizal branches in the same manner as the root tips. The mycorrhizal branches used in experiment 1 were small, usually consisting of only two to four tips; but those used in experiment 2 consisted of large coralloid masses. The root segments were placed in distilled water contained in open specimen dishes. Those to be treated with respiration inhibitors were placed in the desired concentrations of these inhibitors for an hour to allow the inhibitors to penetrate the roots and reduce metabolic activity before the roots were placed in solutions containing phosphorus. They were then transferred to other dishes containing the same concentration of inhibitor, plus sufficient P^{32} to produce an activity of about 300 microcuries per liter. The control segments were placed in distilled water during the conditioning period, then transferred to distilled water plus P^{32}, adjusted to the desired pH. The concentrations of inhibitors used were based on observations of KELLY (**4**), LATIES (**6**), and STENLID (**8**). Stenlid observed that sodium azide completely inhibits respiration of barley and wheat roots at pH 4.5, but produces no inhibition at pH 7.0. The writer likewise found in preliminary experiments that azide inhibits accumulation of P^{32} to a greater extent at pH 4 to 5 than at higher pH values. LATIES (**6**) found inhibition of respiration of barley roots by malonic acid and iodoacetic acid to be much greater at pH 4 to 5 than at higher values. He attributed this to better penetration of the inhibitors in more acid solutions. The pH of the solutions used in these experiments were therefore adjusted by adding dilute NaOH to the malonic acid and dilute HCl to the other solutions. Although the solutions were left unbuffered to avoid adding phosphate in addition to that in the radioactive solutions, little change in pH occurred during the experiments.

The roots were left in the solutions in shallow, open dishes for three hours, since Laties' data indicate that maximum inhibition of respiration usually occurs within three hours. The temperature varied from 25 to 30° C in various experiments. After three hours the roots were removed, rinsed for 30 seconds in distilled water, rinsed for a few seconds in 0.001 *N* H_3PO_4, and again rinsed in distilled water. Rinsing in acid was intended to remove adsorbed radioactive phosphate ion from the surface of the roots by exchange with that in the dilute acid. The terminal centimeter of each root tip was cut off and retained for counting. The root segments and tips were allowed to dry overnight at room temperature, and the activity of each segment was then measured with a Geiger-Muller counter. The results of two experiments with three inhibitors at different pH values are summarized in table I. Inspection of the data for experiment 1 indicates that malonic acid slightly inhibited accumulation of P^{32}, but a statistical analysis of the data revealed that this apparent small inhibition was insignificant. The differences between mycorrhizal roots and nonmycorrhizal root tips proved to be significant, indicating that accumulation by mycorrhizal roots is inhibited to a lesser extent than it is in nonmycorrhizal roots. The results of experiment 2 indicate that the inhibiting effect of azide is decreased at a higher

TABLE I

EFFECT OF RESPIRATION INHIBITORS ON ACCUMULATION OF P^{32} BY PINE ROOTS. RESULTS ARE AVERAGES OF 12 SAMPLES IN EACH TREATMENT.

Treatment	Root tips		Mycorrhizal segments	
	Counts/min.	Activity as % of control	Counts/min.	Activity as % of control
Experiment 1 pH 4.7–4.8				
Control	81,713	...	77,306	...
0.025 M Malonic Acid.....	77,330	94.6	67,405	87.2
0.001 M Sodium Azide	308	0.4	965	1.2
0.005 M Sodium Fluoride ..	986	1.2	1,620	2.1
Experiment 2 pH 5.7–5.8				
Control	20,017	...	117,235	...
0.025 M Malonic Acid.....	30,840	154	151,940	130
0.001 M Sodium Azide	3,796	19	44,845	38
0.005 M Sodium Fluoride ..	23,870	119	175,516	150

pH value, but accumulation by nonmycorrhizal roots is still inhibited more than it is in mycorrhizal roots. More surprising is the observation that malonic acid and sodium fluoride increase accumulation of P^{32} at pH 5.7–5.8. This experiment was repeated, and similar results were obtained.

Effects of pH on inhibition of accumulation

In the course of the experiments it became clear that pH has a very marked effect on the inhibitory action of sodium azide, corroborating the observations of Stenlid (**8**) on wheat and barley roots. Some of the data obtained with pine roots are summarized in table II. These data not only show that decreasing inhibition occurs with increasing pH, but that accumulation of P^{32} is consistently inhibited more in root tips than in mycorrhizal roots. The amount of P^{32} accumulated, expressed as a percentage of the control, is two to three times as great in the mycorrhizal roots as in the

TABLE II

EFFECTS OF pH ON EXTENT OF INHIBITION BY 0.001 M SODIUM AZIDE OF ACCUMULATION OF P^{32} BY PINE ROOTS. RESULTS ARE BASED ON AVERAGES OF ALL SAMPLES COUNTED.

Treatment	No. of samples	Root tips		Mycorrhizal segments	
		Counts/min.	Activity as % of control	Counts/min.	Activity as % of control
pH 4.7–4.8	12	308	0.4	965	1.2
pH 5.0	8	458	2.4	2,711	6.2
pH 5.5	8	115	12.0	796	39.0
pH 5.7–5.8	12	2,669	38.0	62,648	87.0

nonmycorrhizal root tips. While the actual values are very small at pH 4.7–4.8, the difference between them is statistically significant.

The results of these experiments support the conclusions of Stenlid and Laties concerning the importance of pH as a factor affecting the degree of inhibition produced by various chemicals. Reference to table I shows that at pH 4.7–4.8 azide and fluoride strongly inhibited the accumulation of phosphorus, but at pH 5.7–5.8 azide inhibited it to a lesser extent and fluoride and malonic acid actually stimulated accumulation. The slight, apparent decrease in accumulation of phosphorus in the presence of malonic acid observed in experiment 1, table I, is not statistically significant. Experiment 2 was repeated, with similar results; hence the increased accumulation of phosphorus in the presence of malonic acid or of fluoride at pH 5.7–5.8 appears to be adequately substantiated. Laties' data indicate that 0.01 M malonic acid does not inhibit respiration of barley roots at about pH 5.6 and slightly stimulates it at pH 6.0, and Henderson and Stauffer also reported evidence of increased respiration of tomato roots in low concentrations of malonic acid. The writer has found no previous mention of the effect of pH on the inhibitory action of fluoride, but its activity seems to be affected as much by pH as is that of other inhibitors. Bonner and Thimann (1) found that fluoride did not strongly inhibit the growth of pea coleoptiles at pH 6.0, but did not study its effect at other pH values. Laties attributes the greater effectiveness of inhibitors at lower pH values to their greater penetration into the tissues. Perhaps at the higher pH value so little malonic acid and fluoride penetrated the pine roots that they stimulated metabolic activity and consequently increased the accumulation of P^{32} instead of depressing it, but there is no proof of this hypothesis.

Retention of P^{32} on root surfaces

In connection with these studies questions arose concerning the amount of P^{32} adsorbed on the surfaces of the root segments by purely physical forces as compared to the amount accumulated within the cells of the roots. To study this, groups of 20 root tips and 20 mycorrhizal segments were selected. One-half of each group was killed by immersion in boiling water, and both the living and the dead roots were then immersed in solutions containing P^{32} for three hours. They were removed and washed in the usual manner, after which half of the living and half of the dead roots were immersed in distilled water for two and one-half hours and all of the roots were then dried and their activity determined. The results are summarized in table III. Although some phosphorus was held on the dead root tips, even after soaking for two and one-half hours, the quantity was negligible compared to the total amount retained by the living roots. A considerably larger quantity was held by the dead mycorrhizal roots, possibly because the fungal hyphae provide more surface on which adsorption can occur, and little of this was lost by soaking in distilled water. On the whole it appears that the amount of phosphorus held by adsorption on the surface of the

TABLE III

RETENTION OF P^{32} ON THE SURFACE BY LIVING AND DEAD PINE ROOTS. EACH VALUE IS AN AVERAGE OF THE COUNTS PER MINUTE FOR FIVE SEGMENTS.

Treatment	Root tips	Mycorrhizal segments
Living roots	26,611	4,728
Living roots soaked	21,920	2,957
Killed roots	903	665
Killed roots soaked	485	598

types of roots used in these experiments is not large enough to constitute a serious source of error.

Discussion

Most important in terms of the original objectives of this study are the relative effects of the inhibitors on accumulation of P^{32} by mycorrhizal and by nonmycorrhizal roots. The experiments were performed at various seasons of the year, using material from the forest as well as from pot-grown seedlings. Large differences occurred in absolute amounts of P^{32} accumulated by different samples, largely because of differences in volume and surface of different samples of root tips and mycorrhizal segments, and possibly because of differences in accumulative capacity of the tissue contained in different samples. Nevertheless, in all experiments with azide the mycorrhizal segments accumulated two or three times as much P^{32} as the root tips. At pH 4.7–4.8 fluoride also inhibited accumulation of P^{32} to a greater extent in nonmycorrhizal than in mycorrhizal roots, but at pH 5.7–5.8 it caused increased accumulation in both types of roots, although to a greater extent in mycorrhizal roots. Malonic acid did not significantly inhibit accumulation of P^{32} in either type of root at pH 4.7–4.8, but increased it above the control rate at pH 5.7–5.8, the increase being greater in nonmycorrhizal roots.

These results are in only partial agreement with those reported for roots of other species. MACHLIS (**7**) found that malonic acid at pH 5.0 and in the concentration used by the writer reduced intake of bromide by barley roots to less than half of his control rate. His results with azide were similar to those of the writer, complete inhibition of salt intake occurring. LATIES (**6**) found that 0.001 M fluoride at pH 5.0 reduced respiration of barley roots to about 20% of the control rate and 0.01 M malonate at pH 4.6–4.7 reduced respiration of barley roots to about 50% of the control rate. In our experiments malonic acid did not significantly reduce P^{32} accumulation at a low pH, and at pH 5.7–5.8 it caused an increase in accumulation. Fluoride almost completely inhibited it at a low pH and increased it at pH 5.7–5.8. HENDERSON and STAUFFER (**2**) found that neither fluoride nor malonate had much inhibitory effect on respiration of tomato roots at pH 5.2–5.8, but a low concentration of malonate appeared to increase respiration somewhat.

Although salt accumulation is not directly proportional to respiration, any treatment which seriously reduces respiration might be expected to reduce salt accumulation. The two processes would not necessarily be reduced to the same extent, however, as Machlis found that a concentration of azide and cyanide which completely stopped bromide accumulation by barley roots only inhibited about two-thirds of respiration, and low concentrations slightly increased respiration, but not bromide intake. Simultaneous measurements of respiration and of P^{32} accumulation would have been desirable, but lack of time made this impossible.

It seems clear that accumulation of P^{32} by nonmycorrhizal roots is reduced to a greater extent by azide, and at a low pH by fluoride, than is accumulation by mycorrhizal roots. Since these inhibitors presumably act through their effects on respiratory enzymes, our results suggest that there are differences in the enzyme systems of mycorrhizal and nonmycorrhizal roots. Azide is supposed to inhibit cytochrome oxidase, while fluoride, according to authors cited by Laties, is said to inhibit enolase and phosphatase. Malonic acid is said to inhibit succinic dehydrogenase. On this basis it might be suggested that in pine roots, as in barley and tomato roots, an enzyme system sensitive to azide plays an important part in respiration, but that it is more important in nonmycorrhizal than in mycorrhizal roots. The same is true with respect to fluoride, at least at pH 4.6, but neither type of root contains an essential enzyme system which is very sensitive to malonic acid. It would be very interesting to study separately the respiratory enzyme systems of nonmycorrhizal pine roots, of mycorrhizal roots, and of the fungus responsible for the development of mycorrhizae.

Summary

A study was made of the effects of various respiration inhibitors on the accumulation of P^{32} by mycorrhizal and nonmycorrhizal roots of loblolly pine. The inhibitors tested were 0.001 M sodium azide, 0.005 M sodium fluoride, and 0.025 M malonic acid. The roots were exposed to the inhibitors for an initial period of one hour, then exposed to inhibitor plus P^{32} having an activity of about 300 microcuries per liter for three hours.

Azide reduced accumulation to a greater extent in nonmycorrhizal than in mycorrhizal roots at all pH values. The inhibition was almost complete at pH 4.7, but much less at pH 5.7. Fluoride also almost completely inhibited accumulation at the lower pH, but increased it above the control rate at the higher pH. Malonic acid did not significantly reduce accumulation at the lower pH, but increased it above control at the higher pH. The pH of the system has a marked effect on the action of all three inhibitors.

In general pine roots seem to react to respiration inhibitors in about the same manner as barley and tomato roots, although barley roots appear to be more sensitive to malonic acid than pine roots.

It was concluded that, although both types of pine roots contain an azide- and a fluoride-sensitive respiratory enzyme system, that of non-

mycorrhizal roots is much more sensitive than that of mycorrhizal roots. It appears that the two types of roots are somewhat different physiologically.

Part of the work reported in this paper was aided by a grant from the Atomic Energy Commission. The author wishes to acknowledge the assistance of Dr. Karl M. Wilbur in the early part of this study and the cooperation of Dr. Philip Handler of the School of Medicine in providing certain facilities.

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INFLUENCE OF ACIDITY, CALCIUM AND MAGNESIUM ON GROWTH OF XANTHI TOBACCO IN WATER-CULTURE

ROBERT A. STEINBERG

Received September 11, 1950

The influence of acidity on the growth of plants in water-culture has been studied by investigators with varying results. Many have reported a beneficial effect of moderate acidity; whereas others have succeeded in demonstrating equally good growth in solutions of widely different hydrogen-ion concentration. Citations to the literature can be found in a paper by the writer (**5**) dealing with the effects of acidity on the calcium and magnesium requirements of Xanthi tobacco in aseptic culture.

The present experiments are in effect a repetition in the greenhouse of those in aseptic culture at 25° C with about 500 foot-candles of fluorescent light. The yield curves for magnesium remained unaltered in the aseptic cultures with increased acidity, whereas those for calcium did not. An increase in the quantity of calcium needed for maximum growth was apparent at higher acidities.

The use in greenhouse experiments of large containers with fixed charges of nutrients, the presence of heavy precipitates, and the need for preparation of the solutions weeks in advance of the growth period all combined to make iron nutrition of the plant unsatisfactory. Preliminary experiments on iron nutrition were therefore necessary and furthermore seemed desirable, since acidity is generally accepted as an important factor in iron nutrition.

A factor in acidity studies generally ignored is the effect of acidity on the toxicity of even small quantities of trace-element impurities unavoidably present in the solution. It is well known that the harmful effects of high acidity may be aggravated by the presence of excess aluminum, manganese (**1**), and vice versa. Work with fungi also has revealed that higher acidity increases the effect of trace-elements on growth (**4**) even in solutions free of all solid materials.

A further factor in studies on iron chlorosis is another effect of the presence of relatively small but still excessive quantities of trace-element impurities. Symptoms of iron chlorosis in plants may be produced by relatively excessive quantities of zinc, copper, manganese, aluminum, cobalt, nickel, etc. (**1, 3**). These facts may be summarized by the statement that excessive quantities of trace elements relative to iron may lead to toxic responses which are further aggravated by increased acidity. These phenomena were assumed to be the basis for the differences in results reported for studies of acidity and iron nutrition by various workers. Special attention, therefore, was given to the effects of iron salt hydrolysis and trace-element level in these studies.

Experimental procedures

Each plant of oriental Xanthi tobacco was grown singly in a 2½-gallon pyrex glass jar with ten liters of nutrient solution and aerated continuously with compressed air by means of sintered, pyrex glass aerators. The solutions were replenished only with distilled water during the course of the experiment. The jars were protected from light with wooden boxes and masonite pressboard lids, the external surfaces of which had been painted a white color. The internal surfaces were coated with spar varnish. Thorough mixing of the solutions with the precipitated salts on the bottom of the jars was brought about twice weekly by vigorous agitation with air.

The cultures were placed in rows ten wide and six deep on the eastern side of the greenhouse. Those to the east (5×6) were used for the magnesium series and those to the west (5×6) for the calcium series. The acid and neutral cultures were alternated from south to north. Increasing concentrations of calcium and magnesium were arranged from east to west. The cultures furthest east were about 30 inches from the glass.

The seedlings for use in the water-cultures were germinated in wooden greenhouse flats of sterilized sand mixed to a light gray color with magnetite. They were watered with a diluted nutrient solution or distilled water as the occasion demanded. When the leaf spread was about 2½ inches the roots were washed and the seedlings transferred to the jars. The plants were harvested at the beginning of blossoming.

The nutrient solution used in the germination flats and the jars contained per liter: calcium nitrate ($4\ H_2O$), 1.44 gm.; magnesium nitrate ($6\ H_2O$), 0.318 gm.; dipotassium phosphate, 0.366 gm.; and ammonium sulphate, 0.082 gm. The standard solution contained nitrogen, 225; potassium, 165; phosphorus, 65; calcium, 245; magnesium, 30; and sulphur, 20 p.p.m. All micronutrients except boron were added as the chloride. The quantities used were iron, 30 p.p.m.; zinc, 0.2 p.p.m.; copper, 0.05 p.p.m.; manganese, 1.0 p.p.m.; and boron, 0.5 p.p.m. The different levels of calcium and magnesium used were prepared by substituting the chlorides of these two elements for the nitrates. Adjustment in initial acidity was with $N/10$ HCl and $N/10$ NaOH. Heavy precipitates were always formed in each jar on mixing the nutrient solution components and still persisted to some extent at the time of harvest.

The magnesium and calcium concentrations selected for use in these series were confined to a narrow range in the vicinity of the maximum yield. Previous trials were necessary to locate the optima for both elements and the concentrations with which deficiency symptoms were to be anticipated. No deficiency symptoms, other than lowered yields, occurred at the concentrations selected for use in the final series.

Weekly pH averages were obtained by mixing samples of equal volume from each of the duplicate jars before determinations with a glass-electrode meter. The solutions were stirred before sampling. Height of the plants

was also recorded weekly, the distance measured being from lid to base of the apical bud or first bald sucker. The weights refer to air-dried plants.

Preliminary experiments for evaluation of iron compounds

Preliminary experiments were performed to determine the best source of iron to be employed. The salts compared included ferric chloride, ferric citrate, ferric ammonium citrate, ferric potassium tartrate, ferric pyrophosphate, and colloidal (dialyzed) iron. The data are summarized in table I, and show no significant qualitative indication of superiority by any one compound; though ferric ammonium citrate gave smaller plants. Ferrous sulphate has also been employed subsequently and gave almost identical results. The rapid formation of a brownish precipitate in each jar would also indicate that no great difference could be expected. It was necessary,

TABLE I

EFFECT OF SOURCE OF IRON SUPPLY AT THE 60 p.p.m. LEVEL ON THE GROWTH IN HEIGHT OF ORIENTAL XANTHI TOBACCO.*

Compound	9 p.p.m. Mg		27 p.p.m. Mg	
	pH = 4.3	pH = 6.5	pH = 4.3	pH = 6.5
	inches	*inches*	*inches*	*inches*
Ferric chloride	42	42	49	47
Ferric citrate	54	58	54	58
Ferric ammonium citrate ...	40	17	48	27
Ferric potassium tartrate ..	53	55	46	58
Ferric pyrophosphate	46	52	46	46
Dialyzed iron	45	43	49	62

* Individual plants.

however, to stir the contents of each jar vigorously at least twice a week. Failure to do so in preliminary tests rapidly led to a check in growth with symptoms of iron chlorosis and those resembling scald.

A special trial in 1948 may be used to illustrate this response (table II). No repetition of this particular experimental design was considered necessary in view of the striking results obtained and the agreement with previous responses. In the acid series the magnesium level plants averaged 15 inches in height, and the calcium level plants 7.2 inches. In the neutral series the magnesium level plants averaged three inches in height, and those of the calcium series, 7.3 inches. These data were obtained under exactly the same conditions as those of experiments tabulated in table III, except for the omission of vigorous semi-weekly stirring of the solution. These results were qualitatively similar to those observed in two years of preliminary study.

Stirring of the solution was considered to have a twofold beneficial effect. It perhaps aided slightly the dissolution of the precipitate, although

the small aeration stream was probably adequate in itself for this purpose. Its major effect was to coat the roots with a layer of the precipitated salts. The results obtained through agitation—that is absence of iron chlorosis and rapid growth—are attributed to improved absorption of minerals.

The reagents employed appeared to be relatively low in trace elements. This is an important consideration in work on iron nutrition of the plant, since the symptoms of iron deficiency can be produced by the presence of

TABLE II

AVERAGE OF THREE REPLICATES OF ORIENTAL XANTHI TOBACCO IN WATER-CULTURE GROWN DURING THE AUTUMN OF 1948 WITH AERATION BUT WITHOUT SEMI-WEEKLY AGITATION.*

Variable component		Acidity		Height of plants	Air-dry weights		
Element	Concentration	Initial	Final		Tops	Roots	Total
	p.p.m.	*pH*	*pH*	*inches*	*gm.*	*gm.*	*gm.*
Mg	3	4.72 ± 0.06	5.04	22.0	8.1	2.4	10.5
	6		5.48	19.7	9.6	3.1	12.7
	9		5.31	19.0	9.6	2.8	12.4
	12		5.17	8.0	8.0	2.5	10.5
	15		5.05	6.2	7.0	1.8	8.8
Mg	3	7.13 ± 0.17	6.68	8.3	3.1	1.0	4.1
	6		7.16	1.8	1.2	0.5	1.7
	9		7.26	1.3	1.2	0.4	1.6
	12		6.57	1.5	1.3	0.7	2.0
	15		6.56	1.7	1.5	0.6	2.1
Ca	25	4.69 ± 0.07	5.18	6.2	3.2	0.3	3.5
	50		5.08	5.3	3.0	0.3	3.3
	100		4.96	8.3	4.3	0.4	4.7
	150		4.89	8.8	4.5	0.5	5.0
	200		5.04	7.3	3.7	0.4	4.1
Ca	25	6.94 ± 0.18	5.57	7.5	3.3	1.0	4.3
	50		5.23	5.0	2.3	0.9	3.2
	100		5.23	7.2	3.2	1.1	4.3
	150		5.16	6.2	2.7	1.3	4.0
	200		5.56	10.8	4.8	1.0	5.8

* Growth period of 60 days.

an excess of zinc, copper, aluminum, and other elements (**1, 3**). Possibly these toxic effects of the trace elements may prove to be additive, and thus necessitate rigorous purification for their prior removal in acidity studies.

Effect of acidity on the calcium and magnesia optima

The average values obtained from a three-year experiment of plants grown at five levels each of calcium and magnesium and at two acidity levels are presented in table III. There were two replications in one of the years and three replications in the other two years. The total dry weight values were subjected to an analysis of variance and appropriate tests of significance were made. There were large differences among the mean dry weights of the three years (65.6, 42.0, and 46.6) which probably are due to

differences in seasonal conditions and the age at harvest in the different years. The average yields per plant in the calcium series were slightly higher (4 gm.) than in the magnesium series. This difference could be due to a positional effect in the greenhouse or to the much higher content of

TABLE III

AVERAGE OF THREE EXPERIMENTS WITH ORIENTAL XANTHI TOBACCO IN WATER-CULTURES GROWN IN THE GREENHOUSE DURING THE AUTUMNS OF 1946, 1947, AND 1949 (47 ± 5 DAYS)*

Variable component		Acidity		Height in inches of plants	Air-dry weight in grams		
Element	Concentration	Initial pH	Final pH		Tops	Roots	Total**
	p.p.m.						
Mg	3	4.66 ± 0.04	5.68	57.1	38.4	3.5	41.9
	6		5.90	60.1	47.7	3.3	51.0
	9		6.19	60.8	48.9	4.8	53.7
	12		6.32	67.2	48.1	5.2	53.3
	15		6.31	53.6	40.9	4.1	45.0
							49.0
Mg	3	6.97 ± 0.04	7.10	57.4	40.2	3.6	43.8
	6		7.46	57.6	40.0	3.8	43.8
	9		7.43	58.8	48.3	3.7	52.0
	12		7.40	56.5	38.8	3.9	42.7
	15		7.62	60.4	45.2	3.9	49.1
							46.3
Ca	25	4.63 ± 0.04	5.57	51.7	41.6	3.9	45.5
	50		5.43	53.8	43.5	4.0	47.5
	100		5.89	60.4	44.2	4.9	49.1
	150		5.53	58.5	53.4	4.6	58.0
	200		5.41	57.2	47.4	4.6	52.0
							50.4
Ca	25	7.06 ± 0.10	6.82	56.6	43.7	3.7	47.4
	50		6.51	51.4	47.5	3.7	51.2
	100		6.42	57.4	51.7	4.5	56.2
	150		7.15	60.1	56.1	4.7	60.8
	200		6.89	56.5	45.8	5.2	51.0
							53.2

*Each value is the average for eight plants taken at the beginning of blossoming.

**Combined total yield of the magnesium series was 5.8% greater under acid than neutral conditions; of the calcium series 5.6% greater under neutral conditions.

chlorine ion as substituent for nitrate. The average yields of the different levels of the minerals increased for the first three levels, reached a maximum on the fourth level and declined on the fifth level indicating an optimum around the fourth level. When taken separately, magnesium yields were optimum at the third level while calcium yields were best at the fourth

level. No significant difference was present in the values for magnesium and calcium requirements at the two levels of acidity. The height data were not analyzed statistically, but the values appear to be relatively similar to those obtained for yields.

Although acidity variation within the range of pH 4.63 to 7.06 did not show statistical significance between the average yields in either the calcium or magnesium series, the average yield was 2.7 gm. higher in the magnesium series at the acid level, and in the calcium series 2.8 gm. higher at the neutral level. Depression in yield under the influence of high acidity has, as already mentioned, been attributed to a diminution in absorption of calcium. These differences although not statistically significant do indicate trends that are in agreement with the work of previous investigators.

Discussion of results

Adequate iron nutrition has always been a matter of some difficulty in water-culture studies. The standard procedure employed is the frequent addition of small quantities of iron salt. The results of these experiments would indicate that this procedure is unnecessary and lacking perhaps in precision. The initial addition of the total quantity of iron needed by the plant is effective if the contents of the jar are vigorously stirred at intervals. Addition of the iron salt and the other components of the solution several weeks before insertion of the seedlings appeared to be without effect even though a heavy precipitate had been formed immediately on mixing. Absorption of iron was as effective in neutral solution as in acid. The heavy coating of precipitate on the roots apparently served as an adequate source of supply. They agree with the results of numerous workers in sand cultures with dissolved and insoluble iron compounds where the roots are in contact with undissolved iron salt.

The use of initial acidities of pH 4.6 and pH 7.0 were without major influence on the absorption of magnesium and calcium as measured by yields, if the precaution was taken of vigorously stirring the solution at frequent intervals. The purpose of the stirring was to insure adequate contact between root and precipitate in the nutrient solution. Nevertheless indications of a definite trend were evident in the somewhat larger yields with increased acidity in the magnesium series; and the decreased yields in the calcium series. These trends agree therefore with the results previously obtained in aseptic culture as indicating the deleterious effect of excessive acidity on the calcium requirement of the tobacco plant.

The minor effects of a variation in acidity in this study are puzzling to some extent in view of the many experimental results in the literature to the contrary. A probable explanation for the differences reported may consist in the omission from consideration of the possible presence of excessive quantities of trace elements in the culture media. The presence of fairly large quantities of zinc, copper, manganese, aluminum, and so on would cause a display of an increased toxicity with increased activity. The pres-

ence of low quantities of trace elements in the reagents employed in these experiments is presumed, since omission of zinc, copper, or boron led to the production of characteristic deficiency symptoms.

The use of hydrochloric acid and sodium hydroxide for adjustment of acidity; and of chlorides of calcium and magnesium gave appreciable and variable concentrations of chloride and sodium ions. Inspection of table III will disclose that wide differences in concentration of these ions were of little influence on the heights and weights of the plants. Though the acid solutions were much higher in chloride and lower in sodium than the neutral solutions, growth was almost identical at the two levels. A similar comparison between the calcium and magnesium series, the former much higher in chloride, will reveal that the plants of the calcium series averaged 51.8 gm. and those of the magnesium series, 47.7 gm. There was therefore a barely significant advantage at the 5% level shown for high chloride. All or part of this difference, as previously mentioned, might be attributable to a position effect in the greenhouse.

Summary

Water-culture experiments with oriental Xanthi tobacco in the greenhouse were carried out to determine the effect of acidity (initial pH 4.6 and 7.0) on the calcium and magnesium requirements of the plant. Trace-element impurities were at a level sufficiently low to permit production of moderate symptoms of zinc, copper, and boron deficiency. Large 2½-gallon containers with a fixed charge were used and water only replenished. No difficulty was experienced with iron nutrition if the solutions were vigorously stirred semi-weekly so that the roots became covered by a heavy coating of the precipitated iron, phosphate, etc. The relative freedom from toxic quantities of trace-element impurities was also conducive to good iron nutrition. The absence of a major effect of acidity on the calcium and magnesium requirements at the 5% level of variance was also attributed to the absence of deleterious quantities of trace-element impurities. A definite trend was indicated by a 5.8% increase in average yield in the magnesium series; and a 5.6% decrease in average yield in the calcium series at the acid level.

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PHOTOSYNTHESIS IN NATURALLY REARED AQUATIC COMMUNITIES

J. VERDUIN

(WITH TWO FIGURES)

Received April 10, 1950

Introduction

The productivity of aquatic communities has been the subject of numerous researches and theoretical analyses in recent years. Direct measurements of photosynthetic activity per volumetric unit of phytoplankton, however, are conspicuously scarce in the literature. Students of aquatic photosynthesis have usually expressed their results as "yield per 10^6 cells" or "yield per liter of natural water." In the present report measurements of photosynthetic rates per unit of phytoplankton volume are presented, and their bearing on aquatic production is discussed.

Methods

The method of measuring photosynthesis described by OSTERHOUT and HAAS (**2**) was modified by using a Beckman pH meter instead of colored indicators for pH determination. The equilibrium equations published by MOORE (**1**) in his graphic method of determining free carbon dioxide, bicarbonate, and carbonate concentrations in natural waters form a basis for determining carbon dioxide removal during photosynthesis (or evolution during respiration) by making pH measurements at the beginning and end of an experiment. The graph in figure 1 shows the relation between carbon dioxide removal and pH-change in water of a given total alkalinity (90 p.p.m.) between pH values of 7.7 to 9.5. When a Beckman pH meter is used the associated CO_2-change can be measured with an error of less than 10%, if the pH-change amounts to 0.30 pH units or more.

The phytoplankton was collected with a 10-liter Juday trap, the samples were transferred to bottles having a volume of about 30 ml. and placed in a dark compartment until the collecting trip was completed (two to four hours). On arriving at the laboratory the samples were diluted with lake water which had been filtered through silk bolting cloth of the same type used on the trap (0.03–0.04 mm.-mesh). The degree of concentration to which the natural populations were subjected varied with their density in the lake. The highest populations observed, 2.5×10^6 standard volumetric units per liter (8×10^3 cubic micra equals 1 unit, see WELCH (**4**)), were made up to concentrations of five times their natural density. The lowest populations observed were made up to 400-fold concentrations. The experimental concentrations, however, were so low that light transmission through a test flask was never more than a few per cent. lower than for a control

flask containing filtered lake water. No attempt was made to exclude zooplankton.

Subsamples of the phytoplankton concentrate were enclosed in 125-ml. Erlenmeyers for the photosynthesis test. The flasks were placed in a tray through which lake water was circulated to maintain them near lake temperature, and were illuminated with fluorescent light of about 400 foot-candle intensity. Tests showed this intensity to be close to the optimum for photosynthesis of the Tabellaria community which developed in the spring of 1949. The pH was determined at the beginning of an experiment, and again after two hours of illumination. If the pH-change was less than 0.3 units the experiment was continued, but was usually ended within four hours because sharp reductions in rate occurred when tests were prolonged beyond four hours; the rates approaching zero after eight hours of illumination. Such samples, however, recovered their initial rate after being stored in the dark overnight.

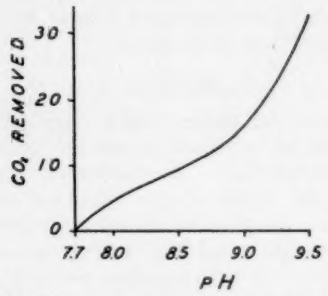

FIG. 1. Relationship of CO_2-removal (micromoles per 100 ml. of water) to pH-change between the values of 7.7 and 9.5, with total alkalinity of 90 p.p.m.

Phytoplankton counts were made, on fresh (unformalinized) samples, soon after the photosynthesis tests were completed. Ten fields were counted for each sample; see WELCH (4) for details of counting procedure. Three-dimensional measurements were made of the major components of the populations studied providing volumetric units for expressing photosynthetic rates.

Results

Measurements of apparent photosynthesis (excess over respiration) were made on plankton samples taken from western Lake Erie at approximately biweekly intervals during 1949. Figure 2 shows the apparent photosynthesis of the phytoplankton from one liter of water graphed against the volumetric units per liter present in the lake. In most cases each datum point represents the average of six determinations, samples collected on the same day from three depths (0-, 5-, and 9-meters) at two stations three miles

apart. The solid line represents the regression line, indicating a rate of 1.5 micromoles of CO_2 absorbed per hour per 10^6 units of phytoplankton. The genera which contributed importantly to these data were Tabellaria, Melosira, Pediastrum, and Fragilaria. The test temperature, being near that of the lake, varied between 1–27° C during the year. The correlation is high (coefficient of .96) but the scatter between 0 and 1 on the abscissa is obscured by the excellent fit on the high values. A separate correlation computed for the scatter diagram between 0 and 1 yielded a coefficient of .92, and a regression line (broken) indicating a rate of 1.1 micromoles of CO_2 absorbed per 10^6 units per hour. The broken line includes significant contributions from zooplankton respiration, which appears as reduced photosynthesis, but zooplankters were conspicuously scarce in the dense Tabel-

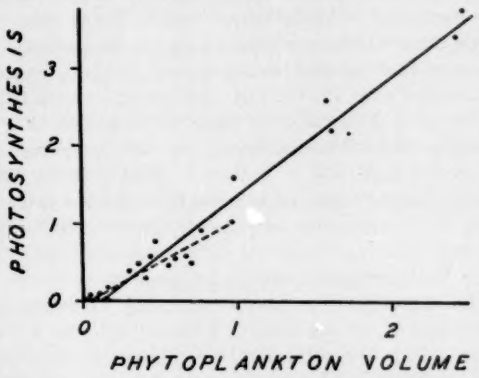

FIG. 2. Photosynthesis exhibited by the phytoplankton from one liter of lake water (micromoles CO_2 absorbed per hour) graphed against the volume of phytoplankton naturally present in a liter of water (standard volumetric units $\times 10^{-6}$).

laria community. It is noteworthy that the highest zooplankton populations present did not exhibit respiration rates which exceeded photosynthesis, but apparent photosynthesis consistently showed positive values. Respiration rates were determined on about two-thirds of the samples studied, making an estimate of the average total photosynthetic rate possible. The apparent rate in 87 tests amounted to 86% of total photosynthesis, so respiration, including that of the zooplankton, averaged only 14% of total photosynthesis. The respiration rate of the dense Tabellaria community, however, which contained negligible amounts of zooplankton, amounted to 12% of total photosynthesis. So the apparent rate of 1.5 micromoles CO_2 absorbed per 10^6 units of phytoplankton per hour must represent a total rate of about 1.7.

Discussion

The method of measuring photosynthesis described above has three advantages which recommend it to students of aquatic photosynthesis: (1) it does not require pretreatment of the water, or elaborate precautions to avoid oxygen contamination, as methods of measuring oxygen evolution do, (2) the measuring technique leaves the samples unchanged so repeated observations can be made on the same sample, and (3) it is a simple, rapid method which requires no elaborate equipment. Although artificial light was used in the present study the method can readily be adapted to experiments under natural light.

The data presented above represent an attempt to measure the photosynthetic activity of the communities arising naturally in western Lake Erie. Other methods have been devised to obtain such information, notably the clear and blackened bottle technique used by RILEY (3). (He has also applied the technique widely in studies of marine production.) The present report affords a marked contrast, in two aspects, to his O_2-production values obtained in Linsley Pond: (1) the high correlation between CO_2-absorption and phytoplankton volume reported here as compared to a statistically insignificant correlation between standing crop and O_2-production in Linsley Pond, and (2) the high rate of apparent photosynthesis (86% of total photosynthesis) reported here, as compared to a negative value for apparent photosynthesis (O_2-consumption exceeded O_2-production) in Linsley Pond. It is obvious that Riley's experiments differ in several respects from those reported here. Both methods, however, are attempts to isolate the primary producers under approximately natural conditions. Whether concentrating the populations and subjecting them to artificial light for a few hours is a greater departure from the natural than confining unconcentrated populations in clear and blackened bottles for seven days is a debatable question. It seems logical, however, to expect that a rather good correlation exists between natural photosynthesis and phytoplankton volume, and that apparent photosynthesis is usually positive in natural waters. If the latter were not true there would be no organic matter deposited on the bottom.

The high correlation evident in figure 2 suggests that the major components of the communities which are adapted to a particular aquatic habitat exhibit closely similar photosynthetic rates per unit of plant volume. This observation has an important bearing on aquatic production. Many students of productivity consider the standing crop of phytoplankton an unreliable index of production. This concept contains the tacit assumption that photosynthetic rates per unit volume vary widely with varying conditions and different organisms. The present study suggests that such variations may not be so erratic as has been assumed, and that the standing crop, since it represents the photosynthetic equipment on which production is based, may be worthy of reconsideration as an index of productivity. The average annual standing crop, at least, when based on a sufficient sampling

frequency and expressed in volumetric phytoplankton units, may provide a fairly satisfactory criterion for estimating the base of the production pyramid.

Summary

1. A method of measuring photosynthesis, using pH-change as a measure of CO_2-removal from natural water, is recommended for studying naturally reared plankton communities.

2. A study of the communities arising in western Lake Erie during 1949 (using fluorescent light of 400 fc) showed a high correlation between volume of phytoplankton and photosynthetic rate, indicating that the major components of these communities were much alike in photosynthetic activity. The total photosynthesis per volumetric unit of phytoplankton was about 1.7 micromoles of CO_2 absorbed per 10^6 standard units per hour (unit is 8×10^3 cubic micra).

3. The data suggest that the standing crop of phytoplankton, when expressed in volumetric units, may be a fairly reliable index of productivity.

The author wishes to acknowledge that the work was facilitated by the encouragement and aid of Dr. T. H. Langlois, director of the Institute, and Edward Kinney, his assistant.

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EFFECTS OF NON-LETHAL CONCENTRATIONS OF 2,4-D ON BUCKWHEAT[1]

D. J. Wort

(WITH TWO FIGURES)

Received June 8, 1950

Introduction

The sequence of events from the time of application of herbicidal concentrations of 2,4-dichlorophenoxyacetic acid until the death of the plant has been elucidated by the use of a variety of plants by numerous investigators (**4, 5** and others). A number of papers concerning the action of non-herbicidal concentrations of the growth-regulator have appeared (**1, 2, 6, 7**) but few have endeavored to trace the changes induced by a series of non-lethal concentrations over a period of time.

In the present study attempts were made to discover the effects of 50, 100, 500, and 1000 p.p.m. 2,4-D on buckwheat, *Fagopyrum esculentum*, variety silver hull, over a time interval of eight days. Observations and analyses for dry weight, total sugars, starch-dextrins, total nitrogen, insoluble nitrogen, and soluble nitrogen were made using plants harvested three, eight and 12 hours, one, two, four, and eight days after spraying.

Materials and methods

Buckwheat, planted in 5-inch pots in the greenhouse, was thinned to two uniform plants per pot. When treated with concentrations of ammonium 2,4-dichlorophenoxyacetate which proved to be non-lethal the plants were 12 to 14 inches high. At this time, April 1, 1949, flower buds were present. A 0.5% aqueous Dreft solution containing 0 (control), 50, 100, 500, or 1000 p.p.m. of the growth-regulator was sprayed on the plants to moisten leaves and stems thoroughly.

Twenty-four control plants (0 p.p.m.) were harvested at the time of spraying. Three hours, eight hours, 12 hours, one day, two days, four days, and eight days after treatment 24 plants from each concentration group, including controls, were harvested and used subsequently to determine dry weight, total sugars, starch-dextrins, and nitrogen values.

In a modified repetition of the experiment, buckwheat was sprayed on July 14, 1949, when 16 to 18 inches tall. About 2% of the plants bore opened flowers. The stems and leaves were thoroughly moistened with an aqueous 100 p.p.m. or 1000 p.p.m. solution of NH_4(2,4-D). The chemical was kindly supplied by the Du Pont Company.

One hundred and twenty control plants (not sprayed) were harvested immediately after the treatment. A similar number of control and treated

[1] This research was made possible, in part, by grants from the Research Fund, University of British Columbia.

plants were harvested 12 hours, one, two, four, and eight days later. In both experiments treatments and subsequent harvestings at one, two, four, and eight days were made at 9 a.m.

Stems and petioles, leaf blades, and roots, were segregated following cleaning, cut into small pieces, heated at 100° C for 30 minutes and then at 75° C until of constant weight. The material was ground to 100 mesh, redried and stored over calcium chloride in desiccators.

Analytical methods

In the April experiment (designated as 49–1 hereafter) 2-gm. aliquots of the dry 100-mesh material were extracted in soxhlets by the procedure given in LOOMIS and SHULL (**3**). Five-gram portions were used in the July experiment (designated as 49–2). Following invertase hydrolysis, total sugars were determined by the semi-micro method of WEINMANN (**8**). The starch-dextrins fraction of the extracted residue was measured by the method of Loomis and Shull, employing fresh saliva. Aliquots of 1 gm. dry weight were used to determine total nitrogen by the standard Kjeldahl procedure. In Experiment 49–2 the powder which had been soxhlet-extracted with 70% ethanol until nitrate- and sugar-free, was used to determine insoluble, or protein, nitrogen. The difference between total N and insoluble N was taken to represent soluble N. In all cases duplicate or triplicate determinations were made of at least two samples. Percentages are based on dry weight.

The value T/C was obtained by dividing the per cent. of a substance in the treated plants by the percentage of that substance in the control plants and multiplying the quotient by 100. In the tables this value was taken to the nearest whole number.

Results and Discussion

Concentrations of 500 and 1000 p.p.m. 2,4-D may be lethal to younger buckwheat plants (**10**), but the more mature plants used in these experiments were less susceptible to the chemical. Even those plants receiving 1000 p.p.m., though badly deformed, were alive eight days after spraying. Generally speaking, the response varied with the concentration used.

By the third hour after spraying the plants which had received 1000 p.p.m. 2,4-D, though turgid, had curved down to rest on their supports. Lesser concentrations caused a slight curvature of stems and curling of petioles by the 12th hour. The severity of bending and coiling increased with time in all cases. At eight days the plants receiving the various treatments formed a series from those appearing almost normal (50 p.p.m.), through increasing deformation (100 and 500 p.p.m.) to plants whose stems were bent and curled and whose petioles were contorted into corkscrew-like coils (1000 p.p.m.).

Leaves produced by a variety of sprayed plants have been reported to be narrowed and decreased in size (**7**). Leaflets often are unable to sepa-

rate, giving pseudosimple or semicompound leaves. The leaves produced by the buckwheat plants sprayed with 500 and 1000 p.p.m. were noticeably smaller than those produced by control plants, but there was no increase in lateral branching or in number of leaves as reported for the bean (**6**). The low concentrations of 1, 5, and 10 p.p.m. 2,4-D used by GREULACH and SINGH (**2**) on beans, produced an increased chlorophyll content. No such increase was observed in buckwheat, in fact, judging from the lighter green color of the treated plants, a decrease in chlorophyll content occurred. There was an increase in anthocyanins in the buckwheat stems, particularly in those plants sprayed with 1000 p.p.m. 2,4-D, similar to the increase reported previously (**10**).

Perhaps because of the age of the plants, no delay in opening of flower buds was evident in plants receiving 50 or 100 p.p.m. 2,4-D. A concentration of 500 p.p.m. delayed the opening several days and 1000 p.p.m. prevented the opening of the majority of the flower buds present at the time of spraying. Spraying beans with 5 p.p.m. 2,4-D delayed flowering by one week (**2**) and 1000 p.p.m. sprays have prevented the opening of flower buds of buckwheat (**10**).

DRY WEIGHT

Within the stems of treated plants there occurred a slight decrease in dry weight T/C values immediately following spraying. A rise to over 100 by the 12th hour was followed by steadily declining T/C values. The pattern in the stems receiving 500 p.p.m. 2,4-D was typical. In this instance the dry weight T/C values were 89, 100, 108, 99, 95, 90, and 68 at three, eight and 12 hours, one, two, four, and eight days, respectively, after spraying. A similar behavior was observed in leaves and roots but the course was slightly more erratic.

As depicted graphically in figure 1, T/C values for stem and leaf dry weight, determined eight days after spraying, decreased with increasing concentration. The values for stems sprayed with 50, 100, 500, and 1000 p.p.m. were 97, 89, 68, and 62, respectively. Leaf values for the same concentrations were 93, 91, 72, and 60. A similar trend was evident in Experiment 49–2. The higher the concentration of 2,4-D used in the spray, the more easily the stems of the buckwheat snapped, suggesting that changes in the cell wall increased with concentration.

TOTAL SUGARS

The T/C values for total sugars in stems and leaves rose above 100 within three hours of treatment with 100, 500, and 1000 p.p.m. 2,4-D (table I). In Experiment 49–1 maxima of 125 to 137 in stem sugars occurred at 12 hours or one day, while in leaves highest values were observed 12 hours after treatment with all concentrations. By the eighth day the values had fallen to the range 48 to 90 in stems and 73 to 95 in leaves. T/C sugar values in roots fell below 100 immediately after spraying and decreased to

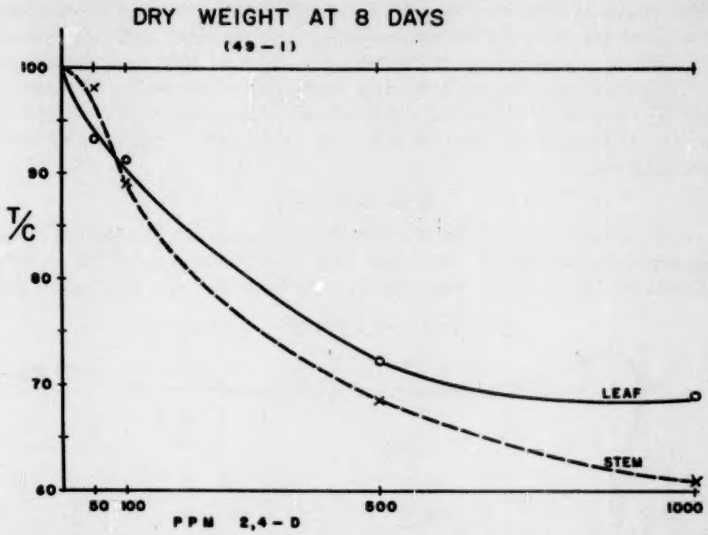

FIG. 1. Effect of concentration of 2,4-D spray on dry-weight T/C values eight days after spraying.

TABLE I

TOTAL SUGAR CONTENT OF BUCKWHEAT SPRAYED WITH 2,4-D*
EXPERIMENT 49-1

	p.p.m. 2,4-D	Time after treatment						
		3 hr	8 hr	12 hr	1 d	2 d	4 d	8 d
Stem	0	6.2%	6.3%	5.3%	5.6%	6.1%	6.2%	7.5%
	50	87	101	137	111	88	105	90
	100	107	118	130	115	107	98	84
	500	...	...	106	135	90	76	53
	1,000	...	...	111	125	91	78	48
Leaf	0	3.2%	3.2%	2.5%	4.5%	4.2%	6.0%	7.8%
	50	116	112	125	73	96	110	95
	100	124	94	125	80	101	70	88
	500	...	...	133	67	74	101	73
	1,000	121	148	160	124	104	69	82
Root	0	1.6%	2.1%	2.0%	2.1%	1.3%	1.2%	2.5%
	50	41	63	49	35	95	79	85
	100	84	46	53	50	76	76	51
	500	55	41	37	...	84	...	51
	1,000	87	43	50	68	80	98	54

*The values given for 0 p.p.m. are per cent. of dry weight. All others are T/C i.e. $\frac{\%\text{ in treated}}{\%\text{ in control}} \times 100$.

final values of 51 to 85. Results substantially similar to those listed above for stems, leaves, and roots were obtained in Experiment 49–2, with values at eight days ranging from 75 for 100 p.p.m. to 44 for 1000 p.p.m. sprayings.

Other investigators (**4, 5, 9**) have obtained similar results. WELLER *et al.* (**9**) obtained no significant difference in reducing sugars of roots and of leaves of treated and control beans but there was a depletion of non-reducing sugars.

STARCH-DEXTRINS

The buckwheat stem starch-dextrins T/C values fell immediately after spraying, but within 12 hours they had risen to maxima of 101 to 111. Thereafter the values declined rapidly. At eight days the T/C values de-

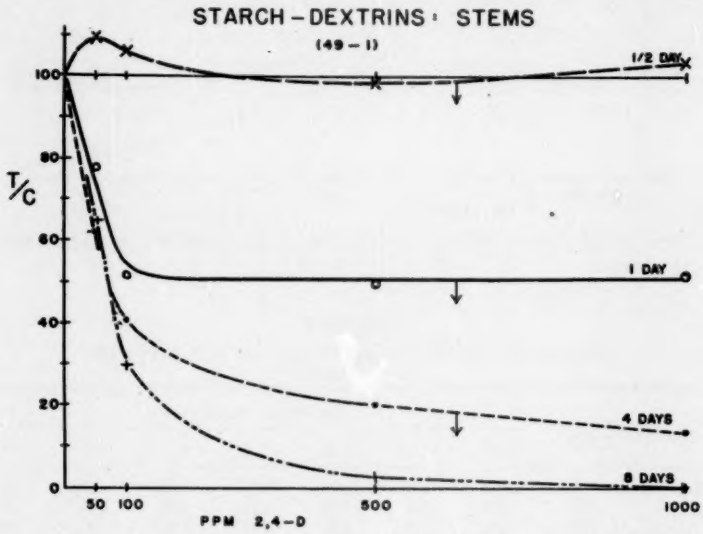

FIG. 2. T/C starch-dextrin values of stems of buckwheat at various times after treatment with 2,4-D. The arrows indicate the direction of movement of the starch-dextrin levels at the time of harvesting.

creased with increasing concentration, being 63, 30, three and zero for 50, 100, 500, and 1000 p.p.m. 2,4-D (fig. 2). Similar changes were found in the second experiment. The results obtained agree substantially with those of others (**4, 5**).

NITROGEN

Total nitrogen T/C values for Experiment 49–1 are given in table II. The values rose steadily in stems and roots in the interval 12 hours to eight days after spraying. Eight days after treatment the values in stems were

TABLE II

TOTAL NITROGEN CONTENT OF BUCKWHEAT SPRAYED WITH 2,4-D
EXPERIMENT 49-1

Time after treatment	Stem Spray concentration p.p.m.					Leaf Spray concentration p.p.m.					Root Spray concentration p.p.m.				
	0	50	100	500	1,000	0	50	100	500	1,000	0	50	100	500	1,000
	%*	T/C**	T/C	T/C	T/C	%	T/C	T/C	T/C	T/C	%	T/C	T/C	T/C	T/C
3 hr.	1.99	106	111	107	102	4.97	106	105	99	99	2.70	102	100	109	96
8 hr.	2.21	102	96	97	97	5.13	96	96	101	99	2.92	86	100	98	89
12 hr.	2.21	89	90	90	104	5.33	95	100	99	97	3.20	86	93	100	93
1 d.	2.18	96	102	105	109	5.40	95	103	99	102	2.60	102	108	100	109
2 d.	2.00	100	114	124	127	5.28	98	98	97	96	3.28	94	103	105	100
4 d.	1.64	130	137	135	168	4.90	101	102	93	96	2.68	114	117	136	137
8 d.	1.50	141	146	149	176	4.62	93	93	93	89	2.67	116	117	134	150

* Based on dry weight.

** $\frac{\%\text{ in treated}}{\%\text{ in control}} \times 100$.

141, 146, 149, and 176 and in roots 116, 117, 134, and 150 for treatment with 50, 100, 500, and 1000 p.p.m. 2,4-D, respectively. In leaves the T/C values for total N remained fairly constant. Similar total N behavior in Experiment 49–2 is evident from table III.

In addition to determination of total N in the dry powder, Experiment 49–2 also measured the N remaining after the powder had been extracted with 70% ethanol until nitrate-free. The N then determined was considered

TABLE III

NITROGEN CONTENT OF BUCKWHEAT SPRAYED WITH 2,4-D
EXPERIMENT 49-2

Days after treatment	0 p.p.m. 2,4-D			100 p.p.m. 2,4-D			1,000 p.p.m. 2,4-D		
	Tot.*	Ins.**	Sol.†	Tot.	Ins.	Sol.	Tot.	Ins.	Sol.
	%	%	%	T/C††	T/C	T/C	T/C	T/C	T/C
				Stems					
½	1.28	0.90	0.38	130	110	175	108	100	121
1	1.30	0.84	0.46	122	120	126	115	123	100
2	1.21	0.74	0.47	126	120	136	140	141	138
4	1.07	0.70	0.37	158	129	210	195	158	162
8	0.80	0.52	0.28	201	214	190	237	287	143
				Leaves					
½	3.68	3.47	0.21	106	106	106	104	99	190
1	3.98	3.77	0.25	115	107	228	107	102	180
2	4.24	3.76	0.48	102	102	96	100	98	119
4	3.70	3.33	0.37	107	108	124	104	97	138
8	3.54	3.12	0.42	104	104	104	88	86	105
				Roots					
½	1.74	1.53	0.21	105	102	124	98	99	95
1	1.75	1.46	0.29	111	117	83	107	109	100
2	1.74	1.51	0.23	105	109	87	115	112	135
4	1.68	1.41	0.27	103	114	41	116	115	126
8	1.54	1.27	0.27	114	137	8	121	144	8

* Total.
** Insoluble in 70% ethanol.
† Soluble in 70% ethanol.
†† $\frac{\%\text{ in treated}}{\%\text{ in control}} \times 100$.

to be protein N. Soluble N values were obtained by subtracting the per cent. insoluble N from the per cent. total N. The values are given in table III.

The changes in protein N values roughly parallelled those for total N, increasing in stems of plants receiving 100 p.p.m. 2,4-D to 214 and in their roots to 137 by the eighth day after spraying. In plants treated with 1000 p.p.m. 2,4-D the values for stems and roots rose to 287 and 144 respectively. Little significant change in protein N values occurred in leaves.

WELLER *et al.* (9) and SELL *et al.* (4) report that, measured six days

after application of 2,4-D, both protein and amino acids accumulated in greater quantities in stems of treated bean plants than in non-treated. Treatment had little effect on the protein and amino acid content of the leaves and roots compared with controls.

An examination of the per cent. N in the stems of buckwheat based upon dry weight reveals that in plants treated with 100 p.p.m. 2,4-D the N-level characteristic of the plants at time of spraying was maintained, while in control plants total N, for example, fell from 1.28% to 0.80% as time progressed. The effect on the T/C value in this instance was produced primarily by the continuous fall in per cent. N in the controls. An increase in per cent. values resulted from treatment with 1000 p.p.m.

Data presented in this paper support the finding that total, protein, and soluble nitrogen contents of stems of treated plants increase over those of controls (**1, 4**), while those of leaves remain relatively unchanged, when measured eight days after spraying. In the experiments with buckwheat, however, increases in total and protein N were obtained in the roots of treated plants. The effect on T/C N values increases with concentration of 2,4-D in the spray.

The experiments described above, involving non-lethal concentrations, and those employing herbicidal doses (**10**) indicate that chemical and morphological responses of buckwheat to various concentrations of 2,4-D are similar to those reported for some other broad-leaf plants.

Summary

Buckwheat was sprayed with 50, 100, 500, and 1000 p.p.m. NH_4(2,4-D). Observations and analyses were made three, eight, and 12 hours, one, two, four, and eight days after treatment.

Generally speaking, the effect of the chemical increased with the concentration used.

The higher the concentration, the greater were deformation, reduction in leaf area, chlorophyll content, and delay or prevention of opening of flower buds. The T/C dry-weight values decreased with increasing concentration and with time.

Total sugar T/C values in stems and leaves rose above 100 within one day after treatment but fell as low as 48 by the eighth day. In roots sugar values fell continuously. Starch-dextrin T/C values in stems fell immediately after spraying, rose within 12 hours to maxima above 100 and thereafter declined rapidly.

T/C values for total N and protein N in stems and roots increased with time and concentration. Little significant change in these values occurred in leaves.

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THE ISOLATION AND PRELIMINARY FRACTIONATION OF PROTEINS FROM DORMANT AND GROWING POTATO TUBERS

J. LEVITT

Received July 5, 1950

In spite of the economic importance of the potato tuber, surprisingly little is known about its proteins (**5**). Osborne and Campbell (see SLACK (**5**)) long ago isolated a component soluble in 10% NaCl which they concluded was a globulin and named tuberin. This was later separated electrophoretically into two components (**5**). It is obvious, however, that there must be more than two proteins present. Since the tuber cells are perfectly normal living cells, they must contain all the essential protoplasmic proteins —structural proteins, nucleoproteins, enzymes, etc. The purpose of this investigation was to obtain some information about these different proteins and to attempt to relate them quantitatively to physiologic changes in the tuber. Russet Burbank potatoes were used in all the experiments described below.

Methods

a) FREEZE DRYING.—In all the above investigations, juice has been obtained from the mash at room temperature, and proteins separated from the juice. In the present investigation the attempt was made to obtain the proteins in as nearly the native state as possible; consequently, a freeze-drying technique was used (**3**). Cohn and his coworkers have shown that this technique does not denature the proteins of blood, with the exception of some lipoproteins (**1**).

With the exception of the grinding, the operations were all performed in a cold room at 0 to 5° C. The tubers were peeled, sliced (about $\frac{1}{8}$″ thick), washed with distilled water at 0 to 5° C, blotted, and frozen in dry ice or liquid air. When frozen solid (about a half hour in dry ice or a few minutes in liquid air), they were placed in vacuum desiccators between layers of anhydrous Al_2O_3 (8 to 12 mesh) and the desiccator immediately evacuated. The desiccators were left evacuated in the cold room for 24 hours. At the end of this time the slices were dry and had been dried in the frozen state. If insufficient Al_2O_3 has been used (when fresh it holds about 10% of its weight of water) or if the desiccator fails to maintain its vacuum, the slices will not dry completely, or in the latter case they may dry after thawing. In this event, they darken in color and fail to retain their original thickness, collapsing toward the middle of the slice. It is therefore easy to detect any such a lack of freeze-drying and the slices are discarded. With a little practice no such difficulties are experienced. After they were dry, the slices were ground in a Wiley mill to pass a 60-mesh screen. This was done at room temperature.

b) EXTRACTION AND PURIFICATION OF PROTEINS.—As heretofore, all operations were performed in the cold room at 0 to 5° C. A 10-gm. sample of potato powder was extracted with 40 ml. solution, larger samples with proportional quantities. The extracting solution consisted of a mixture of .01 M NaCN and .02 *N* KH_2PO_4 in redistilled water. The purpose of the NaCN was to prevent the blackening due to oxidation which otherwise occurred even in the cold. The extract had a pH of 6.9 to 7.1 (determined by a Macbeth pH meter).

The mash was almost immediately filtered through a Buchner funnel covered with Whatman #1 paper. In order to reduce filtration time to a minimum, no further reextraction was attempted. Some 45 ml. of extract was obtained, allowing about 5 ml. for pH determinations. The remaining 30 ml. of filtered extract was poured onto 15 gm. $(NH_4)_2SO_4$ containing enough NH_4OH to obtain the desired pH (about 0.5 ml. of 0.75 *N* for pH values around neutral). When thoroughly mixed, this gave a saturated solution of $(NH_4)_2SO_4$ which was almost immediately filtered through a Buchner funnel covered with Whatman #50 or S and S #576 paper. If the filtrate was not quite clear it was refiltered (sometimes two or three times) through the same paper until absolutely clear. The filter paper was then removed and the precipitate scraped off by means of a stainless steel spatula. It was then resuspended in 10 ml. redistilled water, transferred to a cellophane tube, and dialyzed for two days in several changes of distilled water. Tests with $BaCl_2$ revealed no SO_4 in the dialysate at the end of one day.

In later experiments, the dialysis time was reduced to about four hours as follows. Cellulose acetate strips were cut about 5 mm. wide and 1½ mm. thick. These strips were cemented together (by moistening with acetone) into a flat frame 6 to 8 cm. long and just wide enough to fit snugly into the cellophane dialyzing tubes. The space between the walls of the frame was large enough to accommodate about 5 to 10 ml. solution without causing any appreciable bulge in the tube. Thus diffusion of the $(NH_4)_2SO_4$ occurred over a distance of only 0.75 mm. (from the center of the frame to either wall of the cellophane tube) and four to six changes of water over a period of four hours were sufficient to yield a negative test for $(NH_4)_2SO_4$ with $BaCl_2$. Since complete removal of the last traces of $(NH_4)_2SO_4$ was not required, and since every step that might cause denaturation was avoided, no electrodialysis was used.

The dialyzed suspension was then freeze-dried, giving a yield of about 2% of the potato powder. This dialyzed component of the potato is designated "crude protein."

To determine the nature of the "crude protein," it was analyzed for N, using LINDNER'S (**4**) wet ashing and Nessler technique. Table I shows that the N content was about 12.5%. This value is somewhat low for protein. Soxhlet extractions were therefore made with 1:1 ether-alcohol for 12 hours to determine the lipid content. Values of 3 to 5% of the crude protein were obtained.

TABLE I

PER CENT. NITROGEN IN POTATO POWDER AND CRUDE PROTEIN

Sample	Potato powder	Crude protein	% of total potato N in crude protein
1	1.15	12.25	
2	1.19	12.75	
Av.	1.17	12.50	21.5

All the above results are, however, on the basis of the dry weight of the proteins at room temperature. Since proteins are known to hold water to the extent of about 10% of their weight, even at 100° C (**2**), and since the ash content was not taken into account, the true N content of the crude protein on a water-free, ash-free and lipid-free basis would not be very far from the expected value for pure proteins. Consequently, it seems safe to assume that the crude protein is reasonably free of non-protein components other than lipids and ash.

In order to determine whether or not all of the proteins were extracted by this procedure, the following experiment was performed. A portion of potato powder was analyzed for N. Two portions were then extracted according to the above procedure. The residue was then dried and analyzed for N.

The per cent. of insoluble nitrogen was calculated as follows:

$$\chi = 100 \frac{\left\{ N_p - \left[(N_p - N_r) \times \frac{100}{100-S} \right] \right\}}{N_p}$$

Where χ = undissolved nitrogen (as % of total N)
S = % extracting solution left in residue
N_r = % N in residue
N_p = % N in potato powder

According to these results (table II), about 10% of the total nitrogen of the potato powder was not extractable. To check on these results, the experiment was repeated, but this time the residue was reextracted with distilled water. Though this left less total nitrogen in the residue (since all the soluble nitrogen was removed), the amount left was still about 10% of

TABLE II

EFFICIENCY OF PROTEIN EXTRACTION AS JUDGED BY N ANALYSES OF RESIDUES

Sample	% N	% of extractant left in residue	% undissolved N (calculated)
Potato powder ...	0.87		
Residue 1	0.21	15	10
Residue 2	0.23	17.5	10

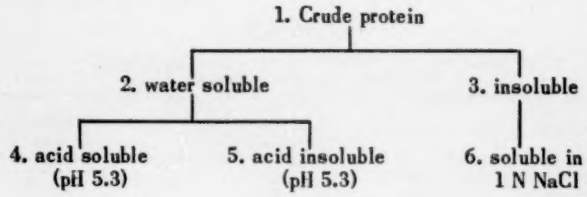
the total N. The same results were obtained when the residue was extracted with M NaCl solution instead of water. It is interesting to note that other workers have reported that about 8% of the total potato protein is insoluble in all the usual protein solvents (5).

Since over 20% of the total N is accounted for by the crude protein (table I), at least two-thirds of the potato protein is extracted by the above procedure, assuming that the insoluble N is protein. However, since the potato does contain some non-protoplasmic protein crystals, the insoluble N may be due to this component. It is also possible that the undissolved N represents soluble protein held in cells that have not been cut open by the Wiley mill. Consequently, it is possible that all or nearly all of the protoplasmic proteins are extractable by the solvent used.

c) Fractionation of proteins.—As long as pH values at or slightly above neutrality were maintained, there was little or no sedimentation of protein during the two days' dialysis. Precipitation was, however, indicated by the marked increase in opacity. Centrifuging for one minute at 600 g failed to throw down any appreciable quantity. Separation into soluble and insoluble components was readily accomplished by filtration through a layer of celite which was first deposited by suction on a sheet of filter paper in a Buchner funnel. The precipitate was then washed with a quantity of water equal to the original volume of suspension. The clear, opalescent, pale yellow filtrate was then freeze-dried. In the dry state it failed to go back into solution in distilled water but readily dissolved in dilute salt solution (*e.g.*, 0.1 M mixture of K_2HPO_4 and KH_2PO_4).

When further fractionation of the soluble protein was desired, KH_2PO_4 was added to the filtrate in sufficient quantity to make it 0.1 *N* and it was then dialyzed. This lowered the pH to about 5.3 and precipitated some of the soluble protein. When dialysis was complete, the precipitate was separated from the soluble fraction by filtration through hard paper. The filtrate was clear and colorless.

Another fraction was obtained by extracting the celite-protein pad with 1 *N* NaCl, filtering, and dialyzing the filtrate. When dialysis was complete, the extract was filtered and both the precipitate (deposited during dialysis) and filtrate were separately freeze dried. The filtrate was combined with the first soluble fraction since it was presumably a small amount of the soluble fraction left in the pad due to incomplete washing. The following is the fractionation scheme:



Protein fractions in dormant and active tubers

As a test of the possible usefulness of the above fractionation procedure, the following experiments were performed to determine whether or not any change in the protein fractions is associated with the breaking of dormancy. Tubers were halved, one half of each tuber placed in a large covered Petri plate at 0 to 5° C, the other half in a similar Petri plate at room temperature (about 25° C). After 10 days, the halves were freeze-dried as described above and shortly after were ground, extracted, and the proteins fractionated into water-soluble and -insoluble components.

Table III fails to reveal any significant difference in the case of tubers that did not sprout (*i.e.*, that were still in their resting period). In the case of tubers that sprouted at room temperature (*i.e.*, that were past their resting period), the water-soluble proteins showed an average increase of 25%.

TABLE III

PER CENT. OF WATER-SOLUBLE PROTEINS IN THE CRUDE PROTEIN OF POTATO HALVES KEPT AT 0 TO 5° C AND 25° C FOR 10 DAYS

Date	0-5° C	25° C
a) New tubers. No sprouting after 10 days at room temperature		
Oct. 27/48	52	55
Nov. 25/48	46.5	40
Nov. 1/49	43.5	46.5
Average	47.3	47.2
b) 1-year-old tubers. Large sprouts after 10 days at room temperature		
May 4/49 (extracted Aug. 22/49)	19	25
Sept. 1/49	31	43
Oct. 1/49	40	45
Nov. 1/49	34	42
Average	31	39

It is interesting to note (table III b, first sample) that even when the powder had been stored for nearly four months before extraction similar relative values were obtained though the total water-soluble fraction was greatly reduced.

In the next experiment, tubers were treated as above and the proteins were further fractionated as described earlier (table IV).

As in the previous experiment, there was a significant increase in the water-soluble proteins at room temperature. The acid-precipitated fraction was definitely higher in the room-temperature halves, and the NaCl-soluble fraction was lower. This may conceivably indicate a conversion of the latter fraction to the former.

It should be pointed out that no direct comparisons may be made between pairs of results obtained at different dates since some differences in technique were deliberately used to find out if this affected the above rela-

tionship; *e.g.*, on January 1 no NaCN was used in the extracting solution. The much higher values for NaCl extract in the February experiment may have been due to the fact that the residue was removed, mixed with the NaCl solution and allowed to extract overnight. In the other two cases, the NaCl solution was merely allowed to filter through the pad over a period of four hours. The March experiment utilized the rapid 4-hour dialysis, the other two the slow 48-hour dialysis. For the two extractions of any one pair, the same technique was always used. The fact that these differences in technique failed to change the relative quantities emphasizes the significance of the correlations obtained.

Thus the fractionation procedure adopted reveals definite protein changes accompanying sprouting of potato tubers. There is an overall increase in water-soluble proteins and a relatively greater increase in those precipitated at pH 5.3. The latter increase may perhaps be at the expense of NaCl-soluble proteins which simultaneously decrease.

TABLE IV

PROTEIN FRACTIONS IN POTATO HALVES KEPT AT ROOM TEMPERATURE AND IN THE COLD (0 TO 5° C) FOR 10 DAYS: CRUDE PROTEIN AS PER CENT. OF POTATO POWDER, ALL OTHERS AS PER CENT. OF CRUDE PROTEIN

Date	Temp.	Crude	Water-soluble	Acid-precipitated water-sol.	Soluble in 1 N NaCl	Total extracted
Jan. 1	5° C	2.20	47.5	negligible	6.3	53.8
	25° C	2.45	51.3	2.1	4.0	55.3
Feb. 1	5° C	2.05	31.7	0.8	28.6	60.3
	25° C	1.95	44.7	3.1	15.9	60.6
Mar. 11	5° C	1.80	41.0	1.65	7.7	48.7
	25° C	1.85	45.7	2.80	6.6	52.3

Summary

1. Proteins were extracted from freeze-dried potato tubers, precipitated and dialyzed. Nitrogen analyses revealed that at least two-thirds of the potato proteins were extracted, comprising about 2% of the total dry weight. The N content of the crude protein was 12.5%, the lipid content 3 to 5%.

2. The crude protein was fractionated into almost equal quantities of water-soluble and -insoluble components. The water-soluble portion was further fractionated by precipitation at pH 5.3. The first water-insoluble fraction was extractetd with M NaCl.

3. Tuber halves that sprouted at room temperature had higher percentages of soluble protein than the control halves kept at 0 to 5° C. Tuber halves that failed to sprout at room temperature (still in their rest period) had the same per cent. of soluble protein as the controls. Still larger relative increases of the acid-insoluble proteins occurred in tuber halves that sprouted. The NaCl-soluble fraction decreased.

This investigation was aided by grants from the University of Missouri Research Council.

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PHYSIOLOGICAL STUDIES OF THE FRUITS OF THE PINEAPPLE [*ANANAS COMOSUS* (L.) MERR.] WITH SPECIAL REFERENCE TO PHYSIOLOGICAL BREAKDOWN

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Received July 17, 1950

Introduction

Physiological breakdown of the fruits of the pineapple [*Ananas comosus* (L.) Merr.] is characterized by a darkening of the flesh of the fruitlets, beginning near their attachment to the core in a region an inch or two above the base of the fruit. The disorder progresses upward and outward, subsequently involving all of the fruitlet except the ovary and a layer approximately one quarter inch deep below the skin. Only in advanced stages do the surface of the fruit and the core become involved. Hence in most fruits there may be no external symptoms of the disorder. Severe cases of physiological breakdown are frequently discovered in pineapples that appear normal and attractive from the exterior. The disorder is not a form of over-ripeness as is true of "internal breakdown" of apples.

One of the commonest causes of physiological breakdown is exposure of the fruits to relatively low temperatures. The disorder has been observed in Florida in the fruits that were induced to ripen during the winter months by forcing the bloom with acetylene gas during "off season." Physiological breakdown has been reported to occur under similar conditions in Australia (**3**). Also pineapples harvested during the summer months may develop breakdown during storage or transit if holding temperatures are too low (**5, 8**), the greener fruits being more subject to the disorder than the riper fruits. Critical temperatures appear to be in the range of 0° to 5° C.

Other conditions than low temperatures may initiate the mechanism causing physiological breakdown. The disorder has been observed in imported fruits that have not been subject to low temperatures during transit. Likewise a somewhat similar if not identical disorder may be induced by heavily waxing mature fruits or by holding them in air-tight containers.

The investigations now being reported were undertaken for the purpose of learning more about the fundamental causes of physiological breakdown with the hope that information so obtained might lead to methods of prevention of this disorder. It was hoped too that the information might be useful in studying physiological disorders of other fruits and of vegetables in cold storage.

Materials and methods

These studies were begun in Florida in 1942 and were transferred to the University of Pittsburgh in 1947. Up to and including 1948 three com-

mercial varieties of pineapples were employed in these investigations—Red Spanish, Cayenne, and Abachi. In 1949 Natal and Queen varieties were included. Thus each of the larger horticultural groups (Spanish, Cayenne, and Queen), as described by HUME and MILLER (4), were represented. (Queen, Natal, and Abachi are listed by the above authors as belonging to the Queen group.)

Studies were first made of the chemistry and physiology of these varieties to determine if there were any varietal characteristics that might be associated with susceptibility to physiological breakdown. The work was subsequently expanded to include observations on physiological and chemical changes produced by subjecting the fruits to storage at low temperatures.

The pineapples were harvested in the "mature green" or "market ripe" stage of maturity and shipped by railway express from Florida to Pittsburgh. This stage is characterized by a uniformly green color, with the eyes or fruitlets well rounded out. It does not include the fruits with a purplish color, such as are often picked for shipments to greater distances. It is realized that pineapples do not attain their maximum content of soluble solids unless permitted to reach the "turning" stage of maturity before picking. However, only fruits in the mature green stage are customarily picked for commercial shipments from Florida and these not only constitute the kinds of fruits subjected to conditions of storage and transport that are conducive to development of breakdown, but they also represent fruits that are typical of those that reach the ultimate consumer.

An entire crate of fruits was employed in each experiment. In instances in which the fruits were rather large, as in the case of Cayennes and Abachi from Miami, the number of fruits per crate was 16 and 18, respectively. In all other lots there were between 25 and 36 fruits in each crate. To compensate for individual variation each fruit was sampled separately and individually analyzed for solids, acids, pH, and ascorbic acid. For peroxidase determinations the juice of four or five fruits was combined.

The fruits were prepared for analysis by peeling, coring and macerating in a Waring Blendor. The juice was filtered through two thicknesses of cheese cloth. Determinations for total soluble solids, acids, pH, and ascorbic acid were made immediately. Samples of juice were placed in a deep-freeze cabinet (−20° C) and held for subsequent enzyme determinations.

Total soluble solids in the juice were determined by means of a specific gravity hydrometer.

In determining total acids, aliquots of the filtered juice were titrated with tenth-normal sodium hydroxide, using phenolphthalein as an indicator. The results were expressed as citric acid.

A Beckman and a Leeds and Northrup pH meter were used for making pH determinations. Each instrument employs a calomel half-cell and a glass electrode.

Ascorbic acid was determined by combining equal portions of juice and 8% acetic acid and titrating with 2,6-dichlorobenzenone-indophenol.

Peroxidase activity was determined after a considerable amount of exploratory work in an attempt to develop a colorimetric method which was both rapid and accurate. The method finally employed was as follows: One ml. of pineapple juice was made up to 100 milliliters with distilled water. A reaction mixture was prepared which consisted of 1 ml. of each of the following: (a) the diluted juice; (b) distilled water; (c) buffer solution (pH 5.2); (d) hydrogen peroxide solution (25×10^{-4} molar); and (e) catechol solution (1%). This mixture was held at 25° C and shaken intermittently for 30 minutes. At the end of this time the reaction came to completion by developing a brown color characteristic of oxidized catechol. The solutions were translucent and the depth of color was measured by means of a Klett-Summerson colorimeter using a blue filter (transmitting at approximately 4200 Å).

It was found that the reaction could be carried out satisfactorily in Van Slyke Cullen tubes, using aeration instead of shaking. This method required 5 ml. of each reagent. In either case the reaction was carried to completion so that the final color did not change, fade, nor darken even after the lapse of 24 hours. A blank determination was always made using distilled water instead of the juice. The enzymatic nature of this process was indicated by the fact that boiled juice gave a negative test for peroxidase.

Results

The results obtained in the studies during 1948 appear in table I. The three varieties, Red Spanish, Cayenne, and Abachi, do not show great differ-

TABLE I

COMPARISON OF CERTAIN JUICE CONSTITUENTS OF THREE VARIETIES OF PINEAPPLES IN THE "MATURE GREEN" OR "MARKET RIPE" STAGE OF MATURITY. SEASON OF 1948.

Variety	Source	Total soluble solids	Total acids	Ascorbic acid
		%	*Gm. per 100 ml.*	*Mg. per 100 ml.*
Red Spanish	Pompano, Florida	10.3	0.92	17.8
Cayenne	Miami, Florida	9.1	0.98	19.7
Abachi	W. Boynton, Florida	9.5	0.71	51.9

ences in regard to total soluble solids and total acids. The solids ranged from 9.1% to 10.3% and the acids were in the range of 0.71 to 0.98 gram per 100 ml. of juice. The Abachi pineapples during this season contained slightly less acid than the other two varieties but this is apparently not a varietal difference because this relationship was not observed in tests made during the following year.

The most striking difference between varieties was to be found in the ascorbic acid content. The Red Spanish, with 17.8 and the Cayenne with 19.7 mg. per 100 ml. of juice, were quite similar, while the Abachi pineapple

contained an average of 51.9 mg. per 100 ml. of juice. Although individuals of the Abachi variety showed considerable variation in ascorbic acid, the difference between this and other varieties was highly significant.

During the season of 1949 the number of lots of pineapples was increased. The list included Cayennes, two lots of Abachi, and one of Queen and Natal, all from Florida. Toward the close of this season a shipment of Cayennes from Mexico and Red Spanish from Puerto Rico were received. The results obtained during 1949 are shown in table II.

The Natal variety differed from the others in having 16.2% solids. As would be expected, the juice of this variety was very sweet. Both Natal and Queen resemble the Abachi variety in external appearance and in possessing a yellow flesh, although the Abachi has a more delicate texture than the other two. Soluble solids in all the other varieties ranged from 9.55% to 13.18%. These values, together with the differences in acids, would

TABLE II

COMPARISON OF CERTAIN JUICE CONSTITUENTS OF FIVE VARIETIES OF PINEAPPLES IN THE "MATURE GREEN" OR "MARKET RIPE" STAGE OF MATURITY. SEASON OF 1949.

Variety	Source	Total Soluble solids	Total acids	pH	Ascorbic acid	Peroxidase activity
		%	*Gm. per 100 ml.*		*Mg. per 100 ml.*	*(Readings on Klett Colorimeter)*
Cayenne	Miami, Fla.	9.55	0.96	3.49	15.0	92
Abachi	Miami, Fla.	11.90	1.03	3.54	43.1	27
Abachi	Ft. Pierce, Fla.	13.18	0.86	3.56	54.8	61
Queen	Ft. Pierce, Fla.	12.75	0.92	3.60	35.8	44
Natal	Miami, Fla.	16.20	0.96	3.11	10.0	85
Red Spanish	Puerto Rico	9.75	0.58	3.99	19.5	260

constitute differences in dessert quality but it is doubtful if they constitute significant varietal differences in all cases because there are so many factors that affect the per cent. of total soluble solids in pineapples.

The Abachi pineapples from Miami, Florida, contained higher acid (1.03 gm. per 100 ml.) than the same variety grown in Ft. Pierce and was higher than all other lots. The other Florida lots of fruits were fairly similar in acid content, ranging from 0.86 to 0.96 gm. per 100 ml. It should be noted that pH varied considerably but did not appear correlated with total acids.

As in the previous season, the ascorbic acid showed the greatest variation. Both lots of Abachi pineapples were high in ascorbic acid. Those from Ft. Pierce contained 54.8 mg. per 100 ml. and those from Miami contained 43.1 mg. per 100 ml. In all of the studies thus far conducted the Abachi pineapple has contained a greater content of ascorbic acid than any of the other varieties. It is apparent therefore that this is a varietal characteristic. The Queen variety was next highest with 35.8 mg. per 100 ml. The

Cayennes from Miami contained 15 mg. ascorbic acid per 100 ml. of juice, being only slightly lower than those analyzed during the previous season.

One of the imported lots of fruits (Red Spanish) was included in this table primarily for the purpose of reporting the peroxidase activity, although solids, acids and ascorbic acid were also included. These data were obtained from the analyses of 12 fruits.

Highest peroxidase activity was shown by Red Spanish pineapples. This variety showed an activity of 260 units on the colorimeter while the other varieties ranged from 27 to 92. Similar results had been obtained in preliminary studies during the previous season. Furthermore a number of random samples of Red Spanish pineapples selected from the local market also showed peroxidase activity higher than any other varieties tested. Red Spanish fruits obtained from the market had been produced in Cuba. It therefore appears that high peroxidase activity is a characteristic of the Red Spanish variety of pineapple irrespective of the area where grown.

During the season of 1949 the experiments were extended to include observations of the effect of low temperature on the physiology of fruits. Upon arrival at the laboratory the fruits in each crate were divided into two lots, each being matched in regard to size and degree of maturity. One lot was held in the laboratory at room temperature (approximately 25° C) for 48 hours and the other was held at 5° C during the same time. (This holding period was selected because it had been found during the work in Florida that physiological breakdown could be produced in green fruits by this treatment.) At the end of the storage period the fruits were held at room temperature until they were fully ripe. The pineapples were analyzed individually as described previously. These results appear in table III.

TABLE III

EFFECT OF LOW TEMPERATURE ON CERTAIN JUICE CONSTITUENTS OF FIVE VARIETIES OF PINEAPPLES

Variety	Storage temperature	Total soluble solids	Total acids	pH	Ascorbic acid
		%	*Gm. per 100 ml.*		*Mg. per 100 ml.*
Cayenne	Room temp.	9.70	0.98	3.51	15.1
"	5° C	10.00	0.94	3.48	15.0
Abachi (Miami)	Room temp.	12.00	1.01	3.68*	40.6
" "	5° C	11.80	1.05	3.46*	45.6
Abachi (Ft. Pierce)	Room temp.	12.34	0.86	3.62*	54.7
" " "	5° C	14.03	0.86	3.49*	55.0
Natal	Room temp.	15.74	0.91	3.81*	8.0
"	5° C	16.66	1.02	3.60*	11.0
Queen	Room temp.	12.75	0.92	3.63	33.7
"	5° C	12.75	0.93	3.57	38.0

*Significant differences.

Storage at the lower temperature produced no significant effects on solids, acids, or ascorbic acid. There were several instances in which the total solids or the acids showed slight differences between the two lots of fruits but these differences were not significant because of the large variation between individual fruits. Likewise the ascorbic acid appears to have increased as a result of the low temperature but here again the results were not statistically significant.

The values for pH were lower in the low temperature lots than in those held at room temperature. This was true for all varieties although statistical significance was shown only in the Abachi and Natal varieties. Thus the pH of the juice of the Abachi pineapples from Miami was 3.68 in those stored at room temperature and 3.46 in those held at 5° C for 48 hours. In the Abachi fruits from Ft. Pierce the pH was 3.62 at room temperature and 3.49 in those held at 5° C. The pH of the Natal fruits held at room temperature and in cold storage was 3.81 and 3.60, respectively. The results for total acids were not as consistent as were those for pH.

Comparison of normal fruits and tissues with those showing breakdown

As mentioned previously, two imported lots of pineapples were received during August of the 1949 season. These arrived after completion of the experiments with Florida-grown fruits and after commercial shipments from Florida had practically ceased. The imported fruits consisted of a crate of Red Spanish from Puerto Rico and a crate of Cayennes from Mexico. One-half of the Red Spanish and all of the Cayennes had physiological breakdown upon arrival. Advantage was taken of this situation to observe the difference between sound and disordered fruits and also between sound and disordered flesh of the same fruits.

In table IV will be found the results of the analyses of the Red Spanish pineapples from Puerto Rico. Each fruit was analyzed separately as was done previously, there being nine sound fruits and 13 showing breakdown. No significant differences between the two lots were shown in regard to

TABLE IV

COMPARISON OF JUICE CONSTITUENTS OF NORMAL RED SPANISH PINEAPPLES WITH THOSE SHOWING PHYSIOLOGICAL BREAKDOWN

Description of fruits	Total soluble solids	Total acids	pH	Ascorbic acid
	%	*Gm. per 100 ml.*		*Mg. per 100 ml.*
Normal fruits*	9.73	0.58	3.90	20.9
Fruits with** physiological breakdown	9.15	0.59	3.98	3.4

* Average of analyses of nine individual fruits.
** Average of analyses of 13 individual fruits.

solids, acids or pH. The ascorbic acid, on the other hand, showed a lower value in the disordered fruits. For example, normal pineapples contained 20.9 mg. per 100 ml. of juice as compared with only 3.4 mg. per 100 ml. in those with physiological breakdown.

The results of comparisons between normal and disordered flesh of two varieties of pineapple are shown in table V. Care was exerted to take the samples of each kind of flesh from comparable areas, that is, the same distance from crown to base, and in most cases analyses were made on opposite halves. This precaution is necessary because there appears to be a decrease in acids and an increase in solids in the flesh of a pineapple when progressing from crown to base.

TABLE V

COMPARISON OF THE JUICE CONSTITUENTS OF NORMAL AND DISORDERED TISSUE OF PINEAPPLE FRUITS

Fruit number	Description of tissues	Variety and source	Total soluble solids	Total acids	pH	Ascorbic acid
			%	*Gm. per 100 ml.*		*Mg. per 100 ml.*
912	Normal	Red Spanish from Puerto Rico	10.30	0.58	3.90	19.3
	Disordered		10.30	0.61	3.92	15.0
925	Normal		9.36	0.60	4.00	17.6
	Disordered		8.02	0.57	4.05	16.0
934	Normal		9.03	0.56	3.95	7.8
	Disordered		9.10	0.59	3.98	1.4
805	Normal	Cayenne from Mexico	11.76	0.87	3.45	5.0
	Disordered		12.96	0.84	3.42	0.9
806	Normal		12.96	0.68	3.48	6.2
	Disordered		10.56	0.62	3.51	1.1
807	Normal		10.56	0.77	3.45	7.6
	Disordered		9.36	0.68	3.40	0.0

In general the results in table V are similar to those in table IV. There was a slightly greater variation in total soluble solids. The decrease in ascorbic acid in the disordered flesh was more pronounced in Cayennes than in Red Spanish. In the former, the differences between sound and disordered fruits ranged from 5.1 to 7.6 mg. whereas in the Red Spanish the differences ranged from 1.6 to 6.4 mg. It is possible that physiological breakdown was further advanced in Cayennes than in the Red Spanish, since all of the Cayennes in the shipment showed physiological breakdown while only half of the Red Spanish fruits were affected.

Oxidizing enzymes

GREEN (2) states that the principal enzymes in plants that cause oxidation of ascorbic acid either directly or indirectly are ascorbic acid oxidase, peroxidase, and polyphenol oxidase. For the past two years in this labora-

tory pineapple juice has been tested for the above enzymes using the method of GERHARDT and EZELL (1) as modified by JIMENEZ (6). In all these tests except one the ascorbic acid substrate was oxidized by the boiled juice and not by the fresh juice, no matter whether the test was made for ascorbic oxidase, peroxidase or polyphenolase. Many tests were made and the reagents were varied so as to cover wide ranges of pH and various concentrations of H_2O_2, all with the same results. Boiled juice was added to the fresh juice to ascertain if an activator were present in boiled pineapple juice as was shown for apple juice by Gerhardt and Ezell. The addition of boiled juice to the fresh juice did not change the results.

During the latter part of November a crate of Natal pineapples arrived from Sebring, Florida. All of these fruits had physiological breakdown. Tests for oxidizing enzymes made with these fruits gave positive results for the first time. Crystalline ascorbic acid was used as a substrate as in the previous tests. The oxidizing power of the juice alone was tested as well as juice plus H_2O_2 and juice plus both H_2O_2 and catechol. The juice alone and the juice plus H_2O_2 and catechol oxidized the ascorbic acid, giving results well above those obtained with boiled juice. This suggests that both ascorbic acid oxidase and peroxidase may be present in the juice. However, these results indicate only trends because there were not enough samples available to afford opportunity for statistical analyses. Furthermore, until additional studies can be made, one cannot be certain whether these values in the enzyme studies were the result of using fresh juice in place of frozen samples, whether these enzymes are more active in winter-ripened fruits than normally ripened ones, or whether some other factors were involved.

Discussion

In considering varietal differences attention should be called to the relatively high content of ascorbic acid in the Abachi pineapple. During the eight years in which these investigations have been conducted the Abachi variety of pineapple has always been found to be higher in ascorbic acid than other varieties under observation. In the results obtained during 1949 the Abachi pineapples averaged 54.8 mg. of ascorbic acid per 100 ml. of juice. A number of individual fruits in the lot were found to range between 60 and 70 milligrams of ascorbic acid per 100 ml. of juice. It must be recalled too that all pineapples employed in these studies had been harvested in the mature green stage of maturity. The author has observed that pineapples harvested in the "turning" stage of maturity contain more ascorbic acid than those picked mature green. On the whole then, the Abachi pineapple can be placed in the same category as citrus fruits as a source of vitamin C. Most of the literature reporting vitamin C content of pineapples apparently refers to other varieties than the Abachi, judging by the relatively low amounts usually reported.

Many of the commercial growers of pineapples consider members of the Queen group (Queen, Natal, Abachi) to be more susceptible than other types to physiological breakdown. The writer is inclined to concur in this

belief. Although breakdown may be rather easily induced experimentally in all types of fruits the disorder appears to occur under normal conditions of handling much more frequently in the Queen type.

In the earlier stages of these studies it appeared as if high ascorbic acid content were associated with susceptibility to physiological breakdown. Thus, two members of the Queen group (Abachi and Queen) are relatively rich in ascorbic acid. Had the Natals likewise contained large quantities of vitamin C then a direct relationship between ascorbic acid and breakdown would be suggested.

It has been reported (**7**) that fruits relatively rich in ascorbic acid are the ones most likely to develop non-enzymatic browning, such as occurs in processed fruits. This type of browning is accompanied by decrease in ascorbic acid content of the product. It has been shown in the present investigation that physiological breakdown in pineapples is accompanied by destruction of ascorbic acid but it has not been definitely established whether the process is enzymatic or non-enzymatic.

It is possible that changes in soluble tannins enter into the development of physiological breakdown. It is known for example that many fruits become less astringent as they ripen because the tannins are rendered insoluble. Tannins are characterized by the presence of phenol groups. It seems that blackening of pineapples during onset of physiological breakdown might be the result of oxidation of these tanins. This is strengthened by the universal observation that green fruits are much more susceptible to breakdown than are ripe fruits.

Storage of pineapples at 5° C for 48 hours resulted in several instances in a lower pH value than in the fruits held continuously at room temperature. It is not known whether this represents a specific physiological effect of low temperatures or whether it merely indicated that ripening was retarded. The percentages of soluble solids and acids of the two lots indicate that they were generally comparable in regard to maturity.

These studies are being continued with greater attention being given to oxidizing and other enzymes.

Summary

A study of the juice constituents of several varieties of pineapples indicated that although soluble solids, pH, and total acids show considerable variation, the varieties show no specific differences unless it might be the Natal, which is usually high in solids.

Ascorbic acid on the other hand is higher in the Abachi and Queen varieties than in Red Spanish, Natal, and Cayenne.

Red Spanish pineapples showed higher peroxidase activity than all of the other varieties employed in these studies.

The only significant differences between constituents of normal fruits and those showing physiological breakdown was the very low percentage of ascorbic acid in the latter.

Subjecting pineapples to 5° C for 48 hours resulted in a significantly lowered pH in the juice of three lots of pineapples.

Tests were made for ascorbic acid oxidase, peroxidase, and phenolase using a modification of the method of Gerhardt and Ezell. Boiled juice oxidized more ascorbic acid than did fresh juice except in the case of Natal pineapples which had ripened in the early winter. The latter were "off season" fruits produced by forcing the bloom with acetylene.

The author is indebted to Miss Harriet Treelisky and Miss Eva E. Schall for assistance with the chemical analyses of the pineapples.

Appreciation is expressed also to the following importers and growers for their cooperation in making the pineapples available for study. The importers were Mr. L. P. Garcia, Tropical Fruits Company, P. O. Box 621, Laredo, Texas; Mr. Frank Maniello, Maniello Brothers and Mayersahn, 324 Washington Street, New York, New York.

The shippers were Miami Pineapple Growers Association, P. O. Box 668, Miami, Florida; Mr. F. G. Palen, Box 111, Pompano, Florida; Mr. P. K. Platts and Sons, P. O. Box 265, Ft. Pierce, Florida; Mr. D. S. Radebaugh, Pineapple Plantations, 413 South Commerce Street, Sebring, Florida; Mr. Oscar Winchester, Box 124, Boynton Beach, West Boynton, Florida.

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THE NUTRITIONAL STATUS OF THE COTTON PLANT AS INDICATED BY TISSUE TESTS[1]

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(WITH ONE FIGURE)

Received July 18, 1950

Introduction

Tissue tests applied to corn and various truck crops have afforded a convenient means of estimating the nutritional status of the plant. The application of suitable tissue tests to cotton should be of similar value. The determination of the portion of the plant to be used, the relation existing between the availability of certain elements and the accumulation of these elements in the sampling tissue, are among the problems which must be solved so that results obtained from tissue tests may be interpreted properly.

Tissue tests developed at Purdue University are being used in diagnoses of nutrient deficiencies in corn (**8, 12, 13, 14**). According to HOFFER (**8**) the best time to make tests on corn is when the plants are well along in maturity. The value of information obtained late in the season is limited, however, by the fact that corrective measures cannot be taken until the following growing season.

CAROLUS (**1**) employed quantitative tissue-testing methods using cabbage and potatoes as his experimental plants. He noted that a deficient supply of nitrogen was reflected in a high concentration of soluble phosphorus and a low concentration of soluble nitrate nitrogen in the experimental tissue. A reverse of the above situation was observed when phosphorus was the limiting element. A potassium deficiency resulted mainly in a low concentration of that element and in some cases was accompanied by increased amounts of soluble nitrate nitrogen. Magnesium deficiency was generally associated with a low concentration of nitrogen. WOLF and ICHISAKA (**16**) drew similar conclusions as to the relationship existing between nitrogen and phosphorus.

EMMERT (**5**) utilized plant tissue tests as a guide in fertilizing tomatoes. He concludes that determinations of soluble nutrients are of greater value as tissue tests than total analyses, which often obscure important variations in the nutrients concerned.

Analytical methods

The tissue to be analyzed was collected in small glass vials, stoppered and held in a refrigerator until extracted. Fresh samples were chopped,

[1] Published with the approval of the Director of the Texas Agricultural Experiment Station as Technical Paper No. 1350.

weighed (1 gram fresh or 200 mg. dry weight), placed in 50-ml. Erlenmeyer flasks, and allowed to stand for 48 hours in 25 ml. of sodium acetate solution (**9**). When dried samples were used it was found that a few milliliters of ethyl alcohol as a wetting agent facilitated the extraction. After the extraction period, measured quantities of activated carbon were added to each flask (**11**). The flasks were shaken and the contents filtered through Whatman's number 12 filter paper. The resulting clear solutions were then used for chemical analysis. Soluble nitrate nitrogen, phosphorus, potassium, calcium, and magnesium concentrations in the cleared extracts were determined by colorimetric and turbidimetric measurements using a Klett-Summerson photoelectric colorimeter. Nitrate nitrogen was determined by a modification of the brucine hydrochloride method as described by PEECH and ENGLISH (**11**). It was found necessary to allow the samples to stand 10 minutes on a steam bath after the addition of the sulphuric acid. Phosphorus was determined by the aminonaphtholsulphonic acid method given by WOLF (**15**).

The sodium cobaltinitrite method for potassium as given by WOLF (**15**) was found satisfactory for this work. The addition of a few drops of a 1% solution of gum arabic to the aliquots taken for the potassium determination stabilized the suspension. Magnesium measurements were made by the titan yellow method and calcium by the ammoniacal citrate procedure as given by PEECH and ENGLISH (**11**).

Experimental

THE SELECTION OF SAMPLING TISSUE

It was necessary at the beginning of this work to determine which portion of the cotton plant would be most suitable for tissue testing. The selection of the tissue to be employed would depend upon: (a) ease of handling, (b) concentrations of the various nutrients within the tissue and (c) response of these concentrations to variations in the substrate.

On June 20, 1947, Stoneville 2–B upland cotton was planted in 3-gallon glazed pots filled with washed creek sand. After emergence of the seedlings one liter of a half strength Hoagland solution (**7**) was applied daily to each pot. On July 3, the plants were thinned to three per pot. From that date on the plants were watered with tap water in which the pH had been adjusted to 5.5 with a few drops of HNO_3 (**4**). On each of the following dates—August 1, 8, 15, and 22—typical plants were harvested and divided into their component parts. Analyses for soluble NO_3 nitrogen, phosphorus and potassium were made and these results are presented graphically in figure 1.

Flowering started on August 10 and it may be observed that this change in the physiological activity of the plant is reflected in reduced concentrations of nitrogen and phosphorus in all tissue analyzed. Except for the main stem petioles, the same was true for the concentration of potassium in the various plant parts (figure 1).

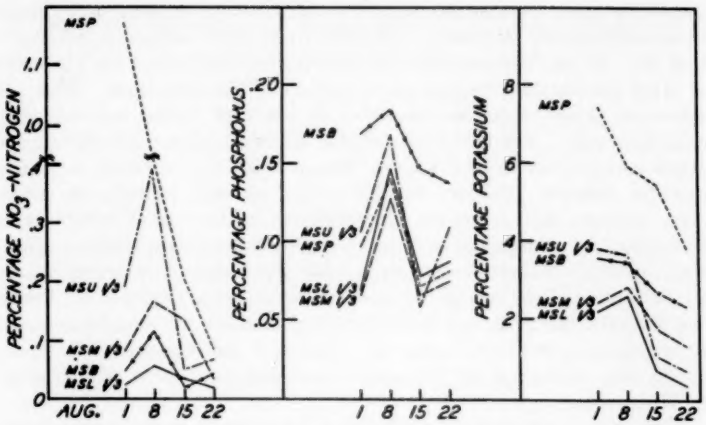

FIG. 1. Soluble NO_3 nitrogen, phosphorus, and potassium in various portions of the cotton plant harvested at weekly intervals. Flowering started August 10. MS, main stem; L, lower; M, middle; U, upper; P, petioles; B, blades.

On examination of the data presented in figure 1, it can be seen that the petioles of the main stem were consistently high in NO_3 nitrogen and potassium. The NO_3 nitrogen of the main stem increased in concentration as the stem apex was approached. The leaf blades were noticeably higher in phosphorus although the petioles maintained a concentration of phosphorus which was favorable for analysis by the procedures employed. As the petioles satisfied the first two criteria for the selection of the sampling tissue set forth earlier in this paper, they were used in further investigations as the experimental portion of the plant.

INFLUENCE OF NUTRIENT SUPPLY ON THE ACCUMULATION OF IONS IN THE PETIOLES

Stoneville 2–B upland cotton was planted in 2-gallon glazed pots filled with washed creek sand in the greenhouse. Eight days after planting the seedlings were thinned to one per pot and the first nutrient applications were made. Nutrients were added every 15 days and the quantities applied were determined by the absorption curves for cotton grown on Cecil sandy loam as found by OLSON and BLEDSOE (10). Nutrient applications were made 15 days in advance of these absorption curves. The plants were divided into 13 groups corresponding to the 13 nutrient treatments employed in this investigation. The base treatment contained twice the quantities of nitrogen, phosphorus, potassium, calcium, and magnesium found in the plants analyzed by Olson and Bledsoe, thus insuring a sufficient supply of nutrients for good growth. Sulphur was added at approximately the same

rate as phosphorus. Finely ground commercial magnetite was added to the sand as a source of iron at the rate of 0.2%. EATON (3) and later CHAPMAN (2) have shown that ample iron is supplied in sand cultures by this method. Other trace elements were applied during the course of the investigation. Plants receiving the base or "high" treatment were supplied with the following total amounts of nutrients during the course of the investigation: NO_3 nitrogen 5.300 gm., phosphorus 1.116 gm., sulphur 1.172 gm., calcium 4.566 gm., potassium 4.174 gm., and magnesium 1.614 gm. The "medium" treatment for each element contained one-half of the quantity of that element in its respective high level. For the "low" treatment each element was dropped separately to one-sixth that quantity contained in the high series. This arrangement gave a high, medium, and low nutrient treatment for each element in which the quantities of the other elements employed did not vary from the base treatment.

This experiment was carried out during the winter of 1947–48 and again in the winter of 1949–50. In the experiment of 1947 the plants were watered carefully to avoid leaching. Although no serious wilting was noted it is felt that insufficient watering accounted for the relatively poor growth obtained. In the 1949 experiment each pot was provided with an enameled pan to catch the leachate, thus making it possible to water the plants freely. The leachate was returned to the pots periodically. Tissue test results of the two experiments closely parallel one another and in view of this only the 1949 data are presented in this paper.

Four tissue harvests were made, at 30, 60, 90, and 145 days after emergence. As the plants were too small at 30 days to provide sufficient petiole material, the entire aboveground portion was harvested. In order to standardize the selection of the sampling tissue, petioles from the third and fourth nodes from the main stem apex were taken on the other harvest dates. The samples were dried and chemical determinations were made as previously described.

Thirty days after emergence, plants receiving the low treatment of nitrogen, phosphorus, and potassium were significantly shorter than those grown in the high series as seen in table I. No real differences were noted in dry weight at this time. Results of the tissue tests on the 30-day sampling are given in table II. The soluble NO_3 nitrogen, phosphorus, and potassium content of the aboveground portion of the plants had significant, positive correlation coefficients when compared with their respective supplies in the substrate. Several interactions were noted at this date. Both the soluble calcium and magnesium content of the plant tops were correlated inversely with the potassium supply. Soluble magnesium in the tops displayed the same correlation when compared with substrate calcium. These observations are in accord with results obtained by GARMAN (6) on the absorption of cations by the cotton plant. Nitrate nitrogen varied directly with the substrate potassium.

The analyses of height measurements taken 60 days after emergence

TABLE I

HEIGHT, DRY WEIGHT AND YIELD AS INFLUENCED BY NUTRIENT TREATMENTS

Treatment	30-Day harvest		60-Day harvest		90-Day harvest	145-Day harvest		Average dry wt. bolls per plant gm.
	Height cm.	Dry wt. gm.	Height cm.	Dry wt. gm.	Height cm.	Height cm.	Dry wt. gm.	
Low nitrogen	15.9*	.390	51.5	5.11**	103	132*	36.1**	29.1*
Med. "	17.9	.460	69.6	9.61	122	131	65.8	59.8*
High "	19.0	.532	64.4	10.90	108	117	72.4	69.2
Low phosphorus	14.8**	.355	56.7	6.66**	129	144**	66.1	60.4
Med. "	17.9	.455	62.4	7.92**	115	125	65.0	65.8
High "	19.0	.532	64.4	10.90	108	117	72.4	69.2
Low sulphur	18.0	.455	62.8	6.72**	117	129	68.1	59.6*
Med. "	18.5	.450	65.4	8.66**	117	129	81.9*	65.5
High "	19.0	.532	64.4	10.90	108	117	72.4	69.2
Low calcium	17.8	.511	68.1	7.83**	125	137**	81.0*	62.2
Med. "	20.2	.509	65.5	10.59	113	122	73.0	62.5
High "	19.0	.532	64.4	10.90	108	117	72.4	69.2
Low potassium	15.8**	.427	61.3	8.34**	109	115	62.4*	59.7*
Med. "	18.4	.567	64.1	7.94	122	131	77.2	68.0
High "	19.0	.532	64.4	10.90	108	117	72.4	69.2
Low magnesium	17.8	.526	65.7	8.45**	124	129	75.8	69.3
Med. "	20.2	.605	71.6	9.56*	112	123	75.4	60.5
High "	19.0	.532	64.4	10.90	108	117	72.4	69.2

* Significant to .05 level.
** Significant to .01 level.

showed no effect of treatment, however, differences in dry weight were highly significant. Table III contains the results of tissue tests on the 60-day petiole samples. No direct comparison can be made between the 30- and 60-day harvests since the entire aboveground portion of the plants were taken on the former date. Soluble NO_3 nitrogen and potassium in the petioles varied directly with their supply in the substrate. Positive inter-

TABLE II

RESULTS OF TISSUE TESTS ON 30-DAY HARVEST
(RESULTS BASED ON OVEN-DRY WEIGHT)

Treatment	Percentage in tops*				
	NO_3 N	P	K	Ca	Mg
Low nitrogen	.138**	.342	4.04	3.40	.363
Med. "	1.187	.323–	3.92	3.71	.541
High "	1.623	.315	4.61	3.46	.536
r†=	.874$	...	...	...	...
Low phosphorus ...	1.520	.172	4.60	3.33	.595
Med. " ...	1.865	.249	3.76	3.44	.539
High " ...	1.623	.315	4.61	3.46	.536
r =	...	.858$	...	...	...
Low sulphur	1.668	.332	4.71	3.56	.645
Med. "	1.376	.340	4.65	3.48	.565
High "	1.623	.315	4.61	3.46	.536
Low calcium	1.462	.268	4.44	3.65	.885
Med. "	1.412	.324	4.62	3.28	.648
High "	1.623	.315	4.61	3.46	.536
r =	...	...	...	...	–.885$
Low potassium	1.225	.321	2.77	4.11	.808
Med. "	1.402	.335	3.84	3.96	.712
High "	1.623	.315	4.61	3.46	.536
r =	.547††	...	.882$	– .771$	–.905$
Low magnesium ...	1.181	.319	4.48	3.57	.520
Med. " ...	1.110	.322	4.47	3.49	.573
High " ...	1.623	.315	4.61	3.46	.536

*Entire aboveground portion of the cotton plant was harvested at this sampling.
**Each figure is an average of six replications.
†r indicates significant correlation coefficients between the concentration of elements in the petioles and nutrient treatment.
††Significant to .05.
$Significant to .01 level.

relations can be seen in table III for petiole potassium and substrate nitrogen on one hand and substrate sulphur on the other. The soluble calcium and magnesium in the petioles again displayed significant negative correlation coefficients when compared with substrate potassium. Petiole calcium had a similar correlation when compared with substrate phosphorus.

Since entire plant samples were not taken at the 90-day harvest, only height measurements were made as an index of growth. No significant effect of treatment on plant height was noted. Chemical analyses of the

petioles showed that each element was correlated directly with its supply in the substrate (table IV). Again negative interactions can be seen in comparing petiole calcium and magnesium with substrate potassium. Petiole calcium was inversely correlated with substrate nitrogen, phosphorus, and sulphur. The inverse interaction between petiole phosphorus and substrate nitrogen is noted in table IV for the first time in the 1949 investigation. This relation was observed in the 60-day harvest of the 1947 experiment.

TABLE III

TISSUE TEST RESULTS ON PETIOLE FROM 60-DAY HARVEST
(RESULTS BASED ON OVEN-DRY WEIGHT)

Treatment	Percentage in petioles				
	NO_3 N	P	K	Ca	Mg
Low nitrogen	.009*	.183	6.45	2.04	.421
Med. "	.631	.131	7.59	1.79	.367
High "	2.210	.173	9.67	1.71	.501
r** =	.887††	...	.898††	...	...
Low phosphorus ...	2.022	.149	8.62	2.84	.536
Med. " ...	2.021	.137	8.13	2.58	.464
High " ...	2.210	.173	9.67	1.71	.501
r =	...	...	...	− .730††	...
Low sulphur	2.124	.157	8.09	2.67	.488
Med. "	1.673	.165	8.99	2.76	.620
High "	2.210	.173	9.67	1.71	.501
r =	...	...	.685†	...	...
Low calcium	2.421	.177	8.13	2.52	.591
Med. "	1.040	.192	7.98	2.32	.504
High "	2.210	.173	9.67	1.71	.501
Low potassium	1.687	.158	4.17	3.16	1.018
Med. "	1.233	.176	6.04	3.05	.648
High "	2.210	.173	9.67	1.71	.501
r =	...	...	.904††	− .801††	− .872††
Low magnesium ...	1.549	.164	8.12	2.89	.465
Med. " ...	2.131	.180	8.33	2.88	.602
High " ...	2.210	.173	9.67	1.71	.501

*Each figure is an average of four replications.

**r indicates significant correlation coefficients between the concentration of elements in the petioles and nutrient treatment.

†Significant to .05.

††Significant to .01 level.

The final harvest was made 145 days after emergence. From table I it can be seen that height and dry-weight measurements were significantly altered by some treatments. Tissue test results on the 145-day harvest are shown in table V. Again, as in the 90-day harvest, the petiole concentrations of each of the five nutrient elements varied directly with their respective substrate levels. Soluble calcium in the petioles was inversely correlated with substrate magnesium. Petiole magnesium varied directly with

the nitrogen and calcium levels of the substrate. Potassium in the petioles showed negative correlations with substrate sulphur and magnesium. Petiole phosphorus varied inversely with substrate nitrogen and calcium. No interactions were noted for petiole nitrogen.

Yield of seed cotton was reduced by the low and medium nitrogen treatments and the low levels of sulphur and potassium. A significant

TABLE IV

TISSUE TEST RESULTS ON PETIOLES FROM 90-DAY HARVEST (RESULTS BASED ON OVEN-DRY WEIGHT)

Treatment	Percentage in petioles				
	NO_3 N	P	K	Ca	Mg
Low nitrogen	.447*	.356	6.10	2.31	.436
Med. "	.610	.258	4.94	1.57	.368
High "	1.042	.218	5.83	1.47	.454
r** =	.918††	−.862††	...	− .817††	...
Low phosphorus ...	1.002	.130	5.42	1.81	.362
Med. " ...	.829	.175	5.41	1.63	.460
High " ...	1.042	.218	5.83	1.47	.454
r =	...	.703††	...	− .682††	...
Low sulphur	.873	.249	4.70	2.60	.431
Med. "	.704	.217	4.86	1.52	.438
High "	1.042	.218	5.83	1.47	.454
r =	...	...	...	− .560†	...
Low calcium	1.036	.245	5.55	1.29	.369
Med. "	.721	.227	4.92	1.39	.400
High "	1.042	.218	5.83	1.47	.454
r =	...	...	...	.606†	...
Low potassium	.703	.211	2.29	2.58	.972
Med. "	.700	.215	4.13	1.85	.624
High "	1.042	.218	5.83	1.17	.454
r =	...	...	.954††	− .916††	−.760††
Low magnesium ...	.911	.204	5.70	1.96	.286
Med. " ...	.758	.207	5.08	1.93	.380
High " ...	1.042	.218	5.83	1.47	.454
r =	...	...	...	...	.621†

*Each figure is an average of five replications.

**r indicates significant correlation coefficients between the concentration of elements in the petioles and nutrient treatment.

†Significant to .05.

††Significant to .01 level.

correlation was obtained (r = .804) when petiole nitrogen of the 90-day harvest from the low, medium, and high nitrogen treatments were compared with the yield of seed cotton for those treatments.

Field Study

After completion of the 1947–48 greenhouse experiment, tissue tests were applied to cotton grown under field conditions. Through the cooperation of

the South Carolina Agriculture Experiment Station tissue tests were run on their field plots at the Pee Dee Experiment Station. These fertilizer plots have been maintained for the past 28 years, and deficiencies are known to exist in nitrogen, phosphorus, and potassium. Petiole harvests were made 90 and 120 days after planting on petioles taken from the third and fourth node from the apex of the main stem.

TABLE V

TISSUE TEST RESULTS ON PETIOLES FROM 145-DAY HARVEST (RESULTS BASED ON OVEN-DRY WEIGHT)

Treatment	Percentage in petioles				
	NO_3 N	P	K	Ca	Mg
Low nitrogen	.0048*	.126	4.37	1.07	.291
Med. "	.0048	.068	4.86	1.10	.620
High "	.0458	.053	4.73	1.03	.794
r** =	.658 ††	−.777 ††	...	...	.909 ††
Low phosphorus ...	.047	.035	4.58	.97	.743
Med. " ...	.070	.052	4.63	1.01	.821
High " ...	.046	.053	4.73	1.03	.794
r =	...	.826 ††	...	...	...
Low sulphur	.102	.059	6.38	1.22	.719
Med. "	.026	.057	4.76	.98	.727
High "	.046	.053	4.73	1.03	.794
r =	...	...	− .569 †	...	...
Low calcium	.056	.075	5.32	.59	.482
Med. "	.029	.071	4.90	.73	.783
High "	.046	.053	4.73	1.03	.794
r =	...	−.960 ††	...	.990 ††	.624 †
Low potassium	.085	.072	.25	1.15	.894
Med. "	.023	.051	3.00	1.07	1.082
High "	.046	.053	4.73	1.03	.794
r =	...	...	.834 ††	.562 †	...
Low magnesium ...	.031	.045	5.63	1.30	.270
Med. " ...	.039	.055	5.03	1.14	.519
High " ...	.046	.053	4.73	1.03	.794
r =	...	...	− .609 †	− .640 †	.921 ††

*Each figure is an average of five replications.

**r indicates significant correlation coefficients between the concentration of elements in the petioles and nutrient treatment.

†Significant to .05.

††Significant to .01 level.

Table VI contains the summarized results of the 90-day harvest. These data confirm to a large extent the results of the previous greenhouse work. The level of soluble NO_3 nitrogen, phosphorus, and potassium within the petioles varied directly with their respective concentrations in the fertilizer; however, interrelations between these elements which influenced petiole concentrations were noted. The negative correlation between substrate nitrogen and petiole phosphorus was apparent. The nitrogen level of the fertilizer

influenced the petiole calcium in an inverse manner; however, the petiole magnesium varied directly with the nitrogen applied. An inverse correlation was shown between phosphorus supply and nitrogen in the petioles, and a direct relation can be seen between fertilizer phosphorus and petiole calcium. This latter relation may be due to the use of superphosphate in the fertilizer. Negative correlations between potassium supplied and the

TABLE VI

TISSUE TEST RESULTS ON PETIOLES HARVESTED FROM 90-DAY OLD FIELD GROWN COTTON (RESULTS BASED ON FRESH WEIGHT)

Treatment*			Yield in lbs. seed cotton per acre	Percentage soluble elements in petioles				
N	P	K		NO_3 N	P	K	Ca	Mg
0	8	4	1,266	.0012**	.0933	.760	.359	.0458
2	8	4	1,674	.0025	.0310	.996	.265	.0358
3	8	4	2,046	.0055	.0200	1.021	.254	.0509
4	8	4	2,376	.0336	.0162	.992	.219	.0511
5	8	4	2,427	.0510	.0132	.799	.258	.0660
6	8	4	2,406	.0927	.0160	.789	.213	.0369
r† =			...	.620 $	−.785 $	...	−.655$	.523 $
4	0	4	546	.1495	.0085	.887	.146	.0532
4	2	4	1,215	.0923	.0087	1.011	.188	.0384
4	4	4	1,806	.0978	.0149	1.976	.212	.0415
4	6	4	1,967	.0855	.0090	1.199	.216	.0382
4	8	4	2,376	.0336	.0162	.992	.219	.0511
4	10	4	2,234	.0625	.0213	1.012	.233	.0492
r =			...	−.438 ††	.555 $	...	.716$	...
4	8	0	665	.1177	.0332	...	.399	.1196
4	8	1	1,412	.0516	.0239	.234	.311	.0859
4	8	2	1,874	.0386	.0249	.599	.314	.0563
4	8	3	2,076	.0388	.0183	.722	.298	.0609
4	8	4	2,376	.0336	.0162	.992	.210	.0511
4	8	5	2,274	.0429	.0180	.828	.239	.0410
r =			...	−.522 $	−.639 $	.808$	−.840$	−.788 $

*1,000 lbs. of fertilizer indicated applied per acre.

**Each figure is an average of four replications.

†r indicates significant correlation coefficients between the concentration of elements in the petioles and nutrient treatment.

††Significant to .05.

$ Significant to .01 level.

petiole calcium and magnesium were obtained. The actual differences in petiole calcium brought about by fertilizer potassium were small, ranging from .399% for the zero potash plot to .239% for the 5% level of potassium. Variations in the petiole magnesium due to fertilizer potassium were of a larger order than those of calcium, as shown in table VI. Inverse relations were obtained between fertilizer potassium and petiole nitrogen and phosphorus. The latter interaction was noted only in the 90-day sampling.

Table VI contains yield data in pounds per acre of seed cotton for each treatment. Too few observations are available to compare yield with petiole nitrogen, phosphorus or potassium. Some generalizations, however, seem obvious. If the levels of NO_3 nitrogen in the petioles of those plants in the nitrogen series are compared with yield, it is seen that plots having an average NO_3 nitrogen content lower than .0055% of the fresh petiole weight produced substantially lower yields. Critical levels for these plants can be observed for phosphorus and potassium in their respective series. In the case of phosphorus this level seems to be in the neighborhood of .016%, and for potassium 0.7% of the fresh petiole weight.

Discussions and conclusions

The petioles of the main-stem leaves most nearly satisfied the requirements of the sampling tissue. The level of soluble nutrients in the petioles reflected the nutrient status of the entire plant. The ease of handling the material, the responses to variations in substrate, and the reflection of interactions between nutrient elements are factors in support of the use of petioles as the experimental tissue. The accumulation of soluble potassium in the petioles is another factor in favor of their selection. It is necessary to standardize as far as possible the collection of petiole material. In these investigations, petioles were collected from the third and fourth nodes from the apex of the main stem.

Highly significant, positive correlations were obtained when the concentrations of soluble nutrient elements in the petioles were compared with their respective levels in the substrate. The age at which deficiencies of nutrients could be detected by tissue tests is difficult to establish. Under favorable growing conditions it may be possible to determine a deficient supply of nitrogen by tissue tests within two or three weeks after emergence. On the other hand, a level of nitrogen which is not sufficient under rapid growing conditions may be ample under conditions not favoring rapid growth. In the 1947–48 greenhouse experiment it is believed plant growth was retarded due to insufficient watering. It is probably for this reason significant correlations between treatment and ion accumulation in the petioles were not obtained until the 90-day harvest. In the 1949–50 greenhouse experiment such correlations were obtained 30 days after emergence when comparing the nitrate nitrogen, phosphorus, and potassium in the tops with their respective substrate levels. The plants of the latter experiment were watered freely and this was reflected in their more rapid growth. Thus the present data indicates that deficiencies of nutrients may be detected by tissue testing methods in advance of the appearance of typical deficiency symptoms. The results of field studies presented here indicated the above conclusion also applies to field-grown cotton as well as greenhouse plants.

The accumulation of nutrient ions in the petioles is complicated by interrelations between these ions. A high concentration of a particular ion in

the petiole does not necessarily indicate an excess of this ion in the substrate but may be associated with a deficiency of some other essential element. A knowledge of these interrelations is of value in the interpretation of results obtained from tissue tests. Petioles of cotton plants grown on low nitrogen substrates were found to contain high amounts of soluble phosphorus. Potassium deficiencies were accompanied by accumulations of calcium and magnesium in the petioles. Substrate calcium and petiole phosphorus seem to be related in an inverse manner as were substrate magnesium and petiole calcium. Other interactions appeared sporadically throughout the course of these investigations but their importance is questionable.

Tissue test may be of considerable value as an early means of predicting yields. In the 1949–50 greenhouse experiment the NO_3 nitrogen content of the tops 30 days after emergence had a correlation coefficient of $r = .852$ when compared with the final yield of seed cotton. This value was significant beyond the .01 level. The 30-day sampling indicated that the NO_3

TABLE VII

CRITICAL CONCENTRATIONS OF PETIOLE NITROGEN, PHOSPHORUS, AND POTASSIUM OBSERVED IN 90-DAY FIELD GROWN AND GREENHOUSE COTTON (RESULTS BASED ON FRESH WEIGHT)

	Percentage in petioles		
	NO_3 N	P	K
Field grown	0.03	.016	0.7
Greenhouse	0.10	.019	0.7

nitrogen content of high-yielding plants should presumably have been above 1.2% and potassium above 3.8% of the dry top weight. The NO_3 nitrogen content in the petioles of these plants remained above 0.7% at the 60- and 90-day harvest, and did not drop below .04% by maturity. The potassium content of dried petioles of high-yielding plants for the 60-, 90-, and 145-day samplings were 6.0, 4.1, and 3.0%, respectively. Direct comparison can be made between the critical concentrations of nitrogen, phosphorus and potassium observed in the 90-day field-grown plants and those noted for the 90-day sampling of the greenhouse experiment by conversion of the latter data to fresh-weight bases. Table VII shows these critical concentrations. A wide difference is noted between the NO_3 nitrogen levels, but it must be remembered that the field-grown plants had other supplies of nitrogen than the nitrates shown by the tests. The phosphorus level of the greenhouse plants appears high. The reduction in yield due to phosphorus in the greenhouse experiment was not statistically significant, thus the .019% phosphorus may be somewhat higher than the true critical concentration.

Summary

Colorimetric and turbidimetric methods were used for the determination of soluble NO_3 nitrogen, phosphorus, potassium, calcium, and magnesium. The procedures were slightly modified to meet the conditions necessary for their application to cotton.

It was found that petioles from the main stem taken from the third and fourth nodes from the apex most nearly satisfied the requirements of sampling.

The level of five nutrients in the petioles reflected the availability of their individual ions in the substrate. Thus, in plants 90 days old positive correlations were obtained when petiole nitrogen, phosphorus, potassium, calcium, or magnesium were compared with their respective concentrations in the substrate. In the 1949–50 greenhouse experiment significant correlations were obtained between tissue concentrations and substrate levels for NO_3 nitrogen, phosphorus, and potassium 30 days after emergence.

A knowledge of interactions between nutrient elements is essential for proper evaluation of tissue test results. Negative correlations were observed between petiole phosphorus and substrate nitrogen, petiole calcium and substrate potassium, and petiole magnesium and substrate potassium. Other interactions were noted.

From these studies it is evident that tissue tests can play an important part in determining the nutritional status of cotton plants. Indications of deficiencies by tissue test can be obtained in advance of the appearance of typical deficiency symptoms.

The writer wishes to express his indebtedness to Dr. Frank M. Eaton and to Dr. Walter E. Loomis for their many helpful suggestions given during the course of this investigation.

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ASCORBIC ACID AND DRY MATTER ACCUMULATION IN TURNIP AND BROCCOLI LEAF DISCS AFTER INFILTRATION WITH INORGANIC SALTS, ORGANIC ACIDS, AND SOME ENZYME INHIBITORS

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(WITH FOUR FIGURES)

Received August 3, 1950

Introduction

Despite the great amount of work that has been done on the ascorbic acid content of various plant tissues very little is known concerning the chemistry of the formation and breakdown of this vitamin in living cells. In order to study these processes in detail it would be desirable to separate them from other processes which occur in the same cells. This poses a rather difficult problem since ascorbic acid is very easily oxidized and may react *in vitro* with many substances which may or may not represent normal reactants in a living cell. Hence the use of living tissues for studying ascorbic acid accumulation is desirable. It should be emphasized that present techniques for using such tissues do not permit a study of ascorbic acid formation or its breakdown, *per se*, but rather a study of the balance between the two processes. However, some tissues make it possible to separate ascorbic acid accumulation to some extent from other processes. Germinating seedlings offer such an approach, one which has been used recently by Mapson *et al.* (**13, 14**). Discs of leaf tissue offer another approach to the problem (**20**). This paper is the second in a series in which discs of leaf tissue have been used to study ascorbic acid accumulation.

Material and methods

Turnip and broccoli plants, varieties Shogoin and Italian Green Sprouting, respectively, were grown in sand culture in the greenhouse. The growing and sampling of the plants, and the methods of measuring the ascorbic acid accumulation in leaf discs, have been described in detail in a previous paper (**20**). Briefly, the technique consists of cutting discs from mature leaves and floating them on the appropriate solutions under the desired conditions. In the present experiments most of the discs were infiltrated prior to being floated. Infiltration was carried out by immersing the sample (about 50 discs, each 1.6 cm. diameter) in the appropriate solution in a 100-ml. beaker. A plug of absorbent cotton was used to hold the discs in place; then the beaker was placed in a desiccator at room temperature and evacuated until the solution boiled vigorously. When air was admitted subsequently to the desiccator the intercellular spaces of the discs were filled with the solution in which they were immersed. Each sample was evacuated three times to give essentially complete infiltration. The discs were

left immersed for two hours; then they were spread out on filter paper under a bank of fluorescent lights. When their intercellular spaces again became filled with air, due to evaporation of water from them, the discs were floated, abaxial side uppermost, on a solution of the same composition used for infiltration.

Subsamples consisting of 15 discs each were analyzed for ascorbic acid initially and after being floated for about 43 hours with continuous illumination. In most cases similar subsamples were dried in a forced-draft oven at 70° C to obtain a measure of the net photosynthesis. The location of the individual crystallizing dishes was randomized under a bank of fluorescent lights. Two or more replicates of each treatment were included in each experiment, and these were arranged under the lights in a randomized block design. The light intensity varied from about 800 to 1000 foot-candles depending upon the location. Temperature control was obtained by placing all of the dishes and the lights in a large room through which air of constant temperature (± 0.5° C) was circulated. The temperature of the solutions varied about 3 to 4° between dishes in any one experiment. The mean temperature in all experiments was about 24° C. Stirring of the solutions as an aid in temperature control was precluded since it was necessary to keep the discs floating to obtain maximum ascorbic acid accumulation. The differences in temperature of the solutions probably resulted from slight differences in evaporation rate from one location to the other. In any case, the randomization minimized errors due to differences in temperature and light intensity. Moreover, previous results (**20**) indicated that the influence of temperature between 24° and 30° C is relatively small, hence precise temperature control was not critical to the success of these experiments.

The presence of persons in the constant-temperature room while setting up an experiment increased the carbon dioxide content of the air to about double its normal value. However, by the end of the experiment the carbon dioxide content decreased to normal values (0.03 to 0.04%). In some of the first experiments the pH of the tissue was estimated by grinding a 15-disc subsample in 15 ml. of distilled water in a small blendor bowl and measuring the pH of the suspension with a glass electrode.

All of the inorganic salts used in these experiments were reagent grade. Of the organic acids, citric and tartaric were reagent grade, acetic was obtained as the C.P. salt of sodium or potassium, and the remainder of the organic acids as well as the 8-hydroxyquinoline, monoiodoacetic acid, and *p*-nitrophenol were obtained from Eastman Kodak Company. Melting points of the Eastman chemicals indicated that they were of satisfactory purity.

Results

Preliminary tests showed that infiltration, either with distilled water or Hoagland's nutrient solution, and subsequent drying, had little or no influence, *per se*, upon ascorbic acid accumulation. Similarly, the discs could be left immersed following infiltration for from one to three hours and the pH

of the nutrient solution could be varied from three to six without appreciably affecting the results.

Infiltration with inorganic salts

Infiltration with 0.02 *N* solutions of various ammonium salts was found to influence the ascorbic acid and dry-weight accumulation markedly. Some typical results are summarized in table I. The pH of the solutions was not adjusted to any predetermined value and the observed pH values ranged from 6.8 to 7.4, except for the distilled water, which was about pH 5.

Visible loss of chlorophyll from the discs was used as a criterion of injury resulting from the treatment. In all experiments, the infiltration with distilled water gave no indication of injury. In the present experiment no injury was obtained with ammonium nitrate; and ammonium sulphate, chloride, and phosphate gave only a small amount of injury. Ammonium

TABLE I

INFLUENCE OF INFILTRATION WITH AMMONIUM SALTS UPON THE RELATIVE ACCUMULATION OF ASCORBIC ACID AND DRY MATTER IN ILLUMINATED LEAF DISCS OF TURNIPS AND BROCCOLI. INFILTRATION WITH DISTILLED WATER = 100

Solutions used	Relative Ascorbic acid accumulation*		Relative Dry weight accumulation*	
	Turnips	Broccoli	Turnips	Broccoli
Nitrate	73	31	37	46
Carbonate	19	−11	58	10
Phosphate ($HPO_4^=$)..	−13	− 8	37	46
Chloride	−22	+ 8	25	39
Sulfate	−23	1	8	34

*The ascorbic acid accumulation and dry weight increase with distilled water were 64 μg/disc and 6.0 mg/disc, respectively, for turnip discs; and 72 μg/disc and 6.1 mg/disc, respectively, for broccoli discs.

carbonate damaged about 5% of the area of turnip leaf discs and about 20% of the area of broccoli leaf discs. Ammonium citrate and ammonium acetate (both 0.02 *N* and pH 7.0) were also included in this experiment, but they proved to be so highly toxic that the results are not reported.

No significant effects of these treatments upon the pH of suspensions of the disc tissue were observed. On the other hand, large differences in ascorbic acid accumulation resulted. Nitrate and carbonate gave somewhat greater accumulation than chloride or sulphate. The greatest accumulation both of ascorbic acid and dry matter occurred in discs infiltrated with distilled water. The ascorbic acid content before infiltration was 73 and 64 micrograms per disc for turnip and broccoli, respectively, and the corresponding dry weights were 6.1 and 6.5 mg. per disc. A comparison of these data with those in table I shows that both the dry weight and the ascorbic acid content approximately doubled in those discs infiltrated with distilled water.

A comparison of the influence of chlorides and nitrates of calcium, potassium, and ammonia upon the accumulation of ascorbic acid and dry matter is summarized in figures 1 and 2. Both turnip and broccoli leaf discs were infiltrated with each salt at each of four concentrations, viz., 0.0025, 0.005, 0.01, and 0.02 *N*. Only one sample was run with each treatment at one time, but the experiment was repeated again three days later. Since the two experiments were randomized independently they were treated as replicates of a single experiment. The summary of the analysis of variance (table II) demonstrates that there were no significant differences between

TABLE II

INFLUENCE OF INFILTRATION WITH SALTS OF CALCIUM, POTASSIUM, AND AMMONIA UPON ASCORBIC ACID AND DRY MATTER ACCUMULATION IN LEAF DISCS OF BROCCOLI AND TURNIPS—SUMMARY OF ANALYSIS OF VARIANCE

Source	DF	Mean squares	
		Ascorbic acid	Dry matter
Replication	1	128.	0.07
Species (S)	1	742. **	20.7 **
Cation (C)	2	10,706. **	16.7 **
Anion (A)	1	14,578. **	5.0 **
C × A	2	273. *	0.5
Concentration (Co)	3	3,625. **	21.0 **
Co × C	6	381. **	2.25**
Co × A	3	368. **	1.22
S × C	2	170.	1.05
S × A	1	179.	0.85
S × Co	3	148.	0.72
Co × C × A	6	62.	0.45
S × C × A	2	64.	0.79
S × Co × C	6	23.	0.24
S × Co × A	3	86.	0.36
S × Co × C × A	6	31.	0.10
Error	47	53.4	...
Error	46	...	0.458

*Significant, $P < 0.05$.
**Highly significant, $P < 0.01$.

the replicates. The difference between species was significant. On an average, the broccoli discs accumulated about 15% more ascorbic acid and dry matter per disc than did the turnip discs, but since none of the species interactions was significant, the results summarized in figures 1 and 2 represent the mean for the two species. In all cases the chlorides gave a lower accumulation of ascorbic acid than the corresponding nitrates. Similarly, ascorbic acid accumulation was greatest in the presence of potassium, least in the presence of ammonia, and intermediate with calcium. The variance associated with ascorbic acid accumulation is contributed largely by these effects of anions and cations. Concentration also was of importance, but the magnitude of all of the interactions was relatively small.

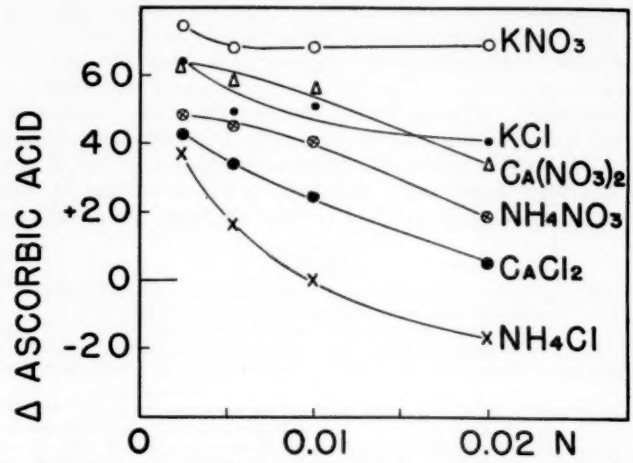

FIG. 1. Influence of ammonium, calcium, and potassium salts upon ascorbic acid accumulation in leaf discs of turnips and broccoli. Each point is a mean value for the two species. Ascorbic acid accumulation is expressed in μg./disc.

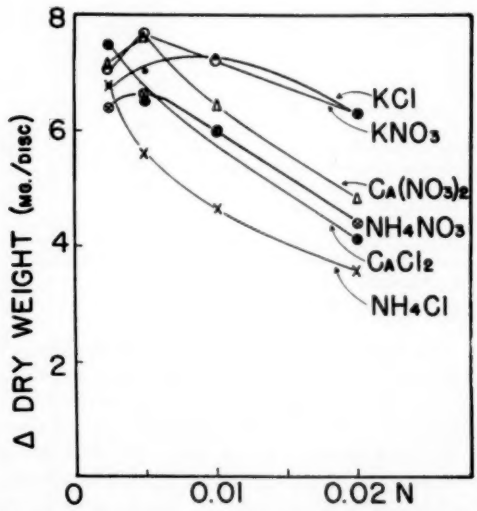

FIG. 2. Influence of ammonium, calcium, and potassium salts upon the accumulation of dry matter in leaf discs of turnips and broccoli. Each point is a mean value for the two species.

Dry-matter accumulation was influenced by the various treatments in much the same manner as ascorbic acid accumulation, although there were some differences in detail. For example, the influence of the anions was relatively less important and the influence of species and the concentration of the solution was relatively more important in the case of dry-matter accumulation than they were in the case of ascorbic acid accumulation. There was little or no visible injury ($< 5\%$ of the area) as the result of any of the treatments in this experiment.

In the above experiments the salts were used individually. That is, any one sample of discs was infiltrated with a solution of a single salt. It seemed desirable to investigate the effect of using various mixtures of anions or cations in a single solution. The first approach used was to combine calcium, potassium, and ammonium ions in a "triangle" arrangement with either chloride or nitrate as a single anion. The concentration of each cation in each treatment is shown in table III. In all treatments the total

TABLE III

CONCENTRATION OF CATIONS IN CHLORIDE OR NITRATE SOLUTIONS USED TO INFILTRATE LEAF DISCS. SEE TABLE IV AND FIGURE 3 FOR A SUMMARY OF THE RESULTS

Treatment No.	Milliequivalents per liter		
	Ca^{++}	K^{+}	NH_4^{+}
1	5	10	5
2	10	5	5
3	5	5	10
4	20	0	0
5	0	20	0
6	0	0	20
7	10	10	0
8	0	10	10
9	10	0	10
10	0	0	0

concentration is 0.02 *N* which is comparable to the concentration of various nutrient solutions (**8**). However, the maximum ammonium concentration (treatment No. 6) is much higher than is ordinarily used for growing plants. Both broccoli and turnip leaf discs were used with three replications of each treatment. The location of the treatments under the lights was randomized separately for each replicate.

The results obtained are summarized in figure 3 and table IV. A summary of the analysis of variance is given in table V. The results obtained with broccoli discs were qualitatively very similar to those obtained with turnip discs so they will not be presented graphically. They differed principally in two respects. The absolute values for the changes in ascorbic acid and dry matter were slightly different and this is shown by the significant

species difference in the summary of variance (table V). Broccoli discs were somewhat less sensitive to chloride, which explains the significant species × anion interaction.

It will be noted (table IV) that the ascorbic acid accumulation was considerably less in the chloride solutions than in the nitrate solutions, particularly with turnip discs. That these effects were of real significance is

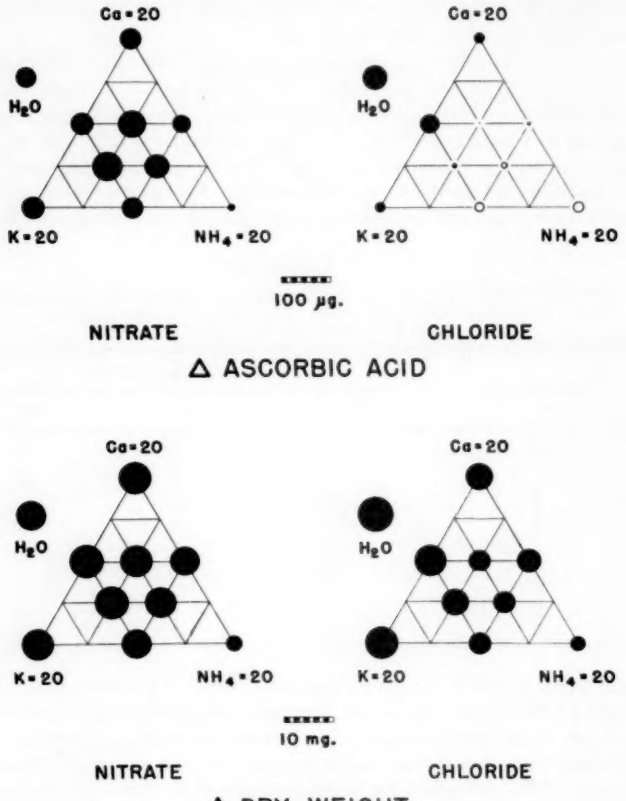

FIG. 3. Influence of various combinations of chlorides and nitrates upon ascorbic acid accumulation and dry-weight increase (net photosynthesis) in turnip leaf discs. The diameter of each circle gives a measure of the change in amount of ascorbic acid or dry matter, as the case may be. An open circle indicates a decrease in amount; solid circle indicates an increase in amount. The magnitude of these changes is indicated by the scales provided in the figure.

The location of each circle in the triangle indicates the cation composition used for that particular treatment. The circles marked H_2O indicate the changes which occurred with discs infiltrated with distilled water.

TABLE IV

MEAN VALUES FOR INITIAL ASCORBIC ACID CONTENT AND DRY WEIGHT OF LEAF DISCS AND THE CHANGE IN THESE CONSTITUENTS DURING ILLUMINATION FOLLOWING INFILTRATION

	Initial		Increase during 42 hours	
	Ascorbic acid	Dry weight	Ascorbic acid	Dry weight
	µg/disc	mg/disc	µg/disc	mg/disc
Turnip discs				
NO_3 triangle	86	5.6	40	5.6
Cl triangle	93	6.2	7	5.8
Broccoli discs				
NO_3 triangle	100	11.3	49	5.0
Cl triangle	125	13.2	29	6.1

indicated by the analyses of variance. Moreover, the various combinations of cations had a highly significant effect upon ascorbic acid accumulation and upon the increase in dry weight. This is evidenced by a general decrease in ascorbic acid and dry-weight accumulation as the concentration of ammonium ions is increased and the concentration of other cations is decreased. This is particularly true in the chloride solution. In the nitrate solutions this effect is less evident and this differential response between chloride and nitrate is reflected by a highly significant cation combination × anion interaction.

Most, if not all, of the solutions used in the above experiment were still not physiologically balanced either with respect to cations or anions. It seemed desirable to test the effect of individual ions in solutions which were

TABLE V

SUMMARY OF VARIANCE FOR ASCORBIC ACID AND DRY WEIGHT ACCUMULATION IN LEAF DISCS INFILTRATED WITH THE SOLUTIONS DESCRIBED IN TABLE III

Source of variance	DF	Mean squares	
		Ascorbic acid accumulation	Increase in dry weight
Replication	2	645**	0.77
Anion (A)	1	21,147**	0.52
Species (Sp)	1	6,886**	11.97 **
Cation Combination (CC)	9	2,091**	12.62 **
CC × A	9	758**	1.37
CC × Sp	9	109	0.40
A × Sp	1	1,300**	7.26 *
A × Sp × CC	9	226	0.05
Error	78	109	1.355

*Significant, P = 0.05.
**Highly significant, P = 0.01.

more nearly balanced. To this end an experiment was devised in which two series of samples were infiltrated with solutions balanced with respect either to anions or to cations. In the latter case, the relative concentrations of cations were maintained in the approximate ratios used in the nutrient solution described by HOAGLAND and ARNON (**8**). The total concentration of the solutions was varied from 0 to 0.01 *N*, but the relative normal concentrations of K^+, Ca^{++}, Mg^{++}, and NH_4^+ were maintained at 7, 8, 4, and 1, respectively. This series was called the "balanced cation" series. All of the cations were provided either as NO_3^-, $SO_4^=$, or $H_2PO_4^-$ salts. For example, in one series solutions were made solely of a mixture of KNO_3, $Mg(NO_3)_2$, $Ca(NO_3)_2$, and NH_4NO_3. The solutions in the other series were all sulphates in one case and all dihydrogen phosphates in the other case. The pH of these solutions was: nitrates, 5.3; sulphates, 5.5; phosphates, 4.3.

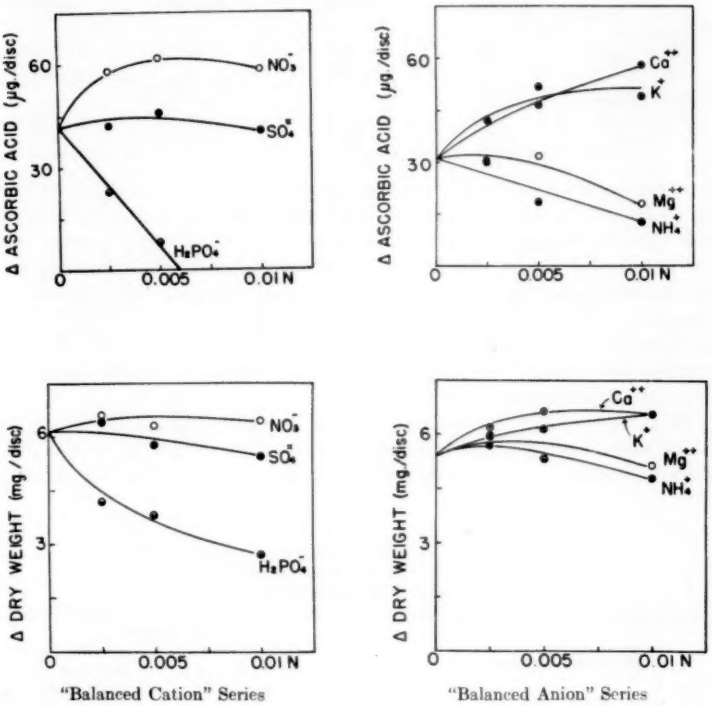

FIG. 4. Influence of various cations and anions upon ascorbic acid accumulation and dry-weight increase in turnip leaf discs.

In the "balanced anion" series the relative concentrations of the anions

were maintained constant. The total concentration of the solutions varied from 0 to 0.01 *N*, but the relative normal concentrations of $SO_4^=$, NO_3^-, and $H_2PO_4^-$ were maintained at 4, 15, and 1, respectively (cf. HOAGLAND and ARNON, 8). All of these anions were provided either as Ca^{++}, K^+, NH_4^+, or Mg^{++} salts. For example, the solution in the Ca^{++} series was composed of mixtures of $CaSO_4$, $Ca(NO_3)_2$, and $Ca(H_2PO_4)_2$. The pH of these solutions were: potassium salts, 5.2; calcium salts, 4.9; magnesium salts, 6.0 and 6.5; ammonium salts, 5.6 and 6.4. The two pH values for the two last-named solutions are those for the two times the experiment was repeated.

Only turnip discs were used and each series was repeated twice. Duplicate samples were analyzed throughout. The results are summarized in

TABLE VI

SUMMARY OF VARIANCE FOR ASCORBIC ACID AND DRY WEIGHT ACCUMULATION IN TURNIP LEAF DISCS FOLLOWING INFILTRATION WITH "BALANCED CATION" AND "BALANCED ANION" SOLUTIONS

Source of variance	DF	Mean squares	
		Ascorbic acid accumulation	Increase in dry weight
"Balanced anion" series			
Cation (CA)	3	1,463 **	3.84**
Concentration (Co)	3	129	0.83
Replication	1	40	8.26
Samples within rep.	1	310	0.01
CA × Co	9	625 **	1.41**
Error	46	129	0.42
"Balanced cation" series			
Anion (A)	2	8,681 **	2.25**
Concentration (Co)	3	1,063 **	1.91**
Replication	1	157	4.75**
Samples within rep.	1	165	0.23
A × Co	6	2,014 **	1.14**
Error	34	57.5	0.19

** Highly significant, P = 0.01.

table VI and figure 4. The initial ascorbic acid contents were 116 and 112 μg./disc in the "balanced cation" and "balanced anion" series, respectively. The corresponding initial dry weight values were 7.6 and 7.1 mg./disc, respectively.

It is interesting to note that when the anions are balanced (fig. 4, right), the type of cation used very significantly influences both ascorbic acid accumulation and the increase in dry weight. Ca^{++} and K^+ both seem to have a stimulatory effect, whereas Mg^{++} and NH_4^+ both appear to depress ascorbic acid accumulation.

Probably the most striking result of this experiment is the marked depressing action of $H_2PO_4^-$ upon ascorbic acid accumulation and, to a much

less extent, upon the increase in dry weight of the discs (fig. 4, left). In the presence of a balance of cations this anion, at its highest concentration, resulted in a loss of 33 μg. of ascorbic acid per disc. In spite of this there was little or no visible damage to the discs with any of the treatments. The effect of phosphate is sharply contrasted to that of sulphate and nitrate. Sulphate was essentially without effect upon either ascorbic acid accumulation or dry-weight increase at any concentration, whereas nitrate apparently stimulated ascorbic acid accumulation slightly.

In a number of experiments it was noted that the accumulation of ascorbic acid and the increase in dry weight seemed to be correlated. This relationship was investigated statistically by pooling the results of a number of different experiments in which the ascorbic acid accumulation was influenced by infiltration with inorganic salts at various concentrations. Included in this were the following ammonium salts: sulphate, chloride, nitrate, carbonate, acetate, and phosphate; the following potassium salts: chloride, nitrate, and acetate; and calcium nitrate and calcium chloride. In addition the results of the cation triangle and the "balanced ion" experiments were included. The results obtained both with turnip and broccoli discs were combined for this calculation. The correlation coefficient calculated between ascorbic acid accumulation and dry weight increase was $r = +0.8331$ ($n = 156$). The regression equation calculated from these data was:

$$Y = 13.83\,X - 40.16$$

where X = the increase in dry weight (mg./disc), Y = the increase in ascorbic acid (μg./disc).

Infiltration with salts of organic acids

The effects produced by infiltrating leaf discs with the sodium salts of organic acids were studied. These salts, adjusted to pH 5.0 ± 0.1 with HCl or NaOH, were used with the infiltration technique described above. Sodium nitrate was included in these studies to provide a basis for comparison between experiments.

The two salts studied most completely were sodium succinate and its homologue, sodium malonate. The results of five experiments with turnip leaf discs are summarized in table VII. At the concentrations reported in this table these salts were not toxic (less than 5% injury). However, sodium malonate was rather toxic at a concentration of 0.02 M. It will be noted that 0.005 M sodium malonate reduced ascorbic acid accumulation rather markedly (to one-third of that obtained with distilled water), whereas sodium succinate at the same concentration was without effect. At concentrations of 0.01 M, sodium malonate actually caused a loss of ascorbic acid from the discs, apparently without appreciable injury to the discs. At this concentration sodium succinate gave a slightly lower ascorbic acid accumulation than the control. The changes in dry weight are similar,

TABLE VII

INFLUENCE OF INFILTRATING TURNIP-LEAF DISCS WITH SODIUM SUCCINATE AND SODIUM MALONATE SOLUTIONS UPON THE RELATIVE ASCORBIC ACID ACCUMULATION AND DRY WEIGHT INCREASE. INFILTRATION WITH DISTILLED WATER = 100

Salt	Relative ascorbic acid accumulation*		Relative dry weight accumulation*	
	0.005 M	0.01 M	0.005 M	0.01 M
Nitrate	132	126	98	95
Succinate	100	76	92	92
Malonate	33	− 7	62	46

*The average ascorbic acid accumulation and dry weight increase with distilled water were 54 μg/disc and 6.1 mg/disc, respectively.

although the relative effect of malonate is smaller in this case and succinate is without significant effect. An analysis of variance showed that the influence of these substances upon both ascorbic acid accumulation and the increase in dry weight was very highly significant ($P < 0.001$).

The results of three experiments in which sodium malate and sodium tartrate were used with turnip leaf discs are summarized in table VIII. The response to infiltration with malate compares reasonably well with that obtained from succinate at the same concentration. Although the data for succinate at a concentration of 0.02 M are not given in table VII, this comparison applies also at that concentration. However, tartrate depresses ascorbic acid accumulation at the higher concentration rather markedly and does so without producing an appreciable amount of visible injury to the leaf tissue. The dry-weight changes are influenced relatively less by tartrate than is ascorbic acid accumulation. An analysis of variance showed the following effects upon ascorbic acid accumulation to be very highly significant ($P < 0.001$): type of anion, concentration of infiltrating solution, and the concentration of solution X type of anion interaction. In the case

TABLE VIII

INFLUENCE OF INFILTRATING WITH SODIUM MALATE AND SODIUM TARTRATE SOLUTIONS UPON THE RELATIVE ASCORBIC ACID ACCUMULATION AND DRY WEIGHT INCREASE IN TURNIP-LEAF DISCS. INFILTRATION WITH DISTILLED WATER = 100

Salt	Relative ascorbic acid accumulation*			Relative dry weight increase*		
	0.005 M	0.01 M	0.02 M	0.005 M	0.01 M	0.02 M
Nitrate	124	119	78	91	88	77
L-Malate	74	65	33	96	88	79
Tartrate	82	37	−57	80	80	41

*The average ascorbic acid accumulation and dry weight increase in discs infiltrated with distilled water were 54 μg/disc and 5.6 mg/disc, respectively.

of dry-matter accumulation the effect of the type of anion was highly significant ($P < 0.01$) and the concentration of the solution was significant ($P < 0.05$).

Citrate has proved to be much more toxic to leaf discs than have the 4–C dicarboxylic acids at the same molar concentration. Mole for mole, acetate has proved to be equally as toxic. At a concentration of 0.01 M both caused appreciable tissue damage (yellowing) in some cases. However, sometimes the injury was not serious at this concentration. At one-half this concentration acetate solutions caused little or no damage but they likewise were without significant effect upon ascorbic acid accumulation. At a concentration of 0.005 M, citrate was observed in one experiment to reduce ascorbic acid accumulation to about one-half that of the controls without any influence upon dry-weight increase and with little or no visible damage to the leaf tissue. In another experiment, at the same concentration, about 10% of the tissue was damaged and both ascorbic acid and dry-matter accumulation was reduced to about one-fourth of that of the control.

Infiltration with enzyme poisons

The high correlation between ascorbic acid accumulation and the increase in dry weight during the period of illumination suggests that these two processes are connected in some way. This is further supported by the earlier observations that carbon dioxide is required for both processes in leaf discs (**20**). The relationship between these two processes has been studied further by infiltrating discs with various enzyme poisons. It must be remembered that in all cases the test period has been in excess of 40 hours, which makes the use of such inhibitors rather difficult. Any drastic upset in various steps in metabolism might be expected to lead to serious injury to the tissue after such a long period of exposure. However, the errors involved in ascorbic acid analysis and the rate of change in ascorbic acid content make a shorter experimental period undesirable. In view of the influence of various anions and cations upon ascorbic acid accumulation a more or less innocuous substance has been included in the experiment each time an enzyme poison has been tested. For example, samples infiltrated with sodium chloride were included in a study of the influence of the enzyme poison sodium fluoride. Control samples infiltrated with distilled water were also included. Furthermore, all of the solutions were adjusted to pH 5.0 ± 0.1 before being used.

Sodium fluoride, at a concentration of 2.5×10^{-3} M, produced little or no injury (less than 3%) and had no influence upon ascorbic acid accumulation or the change in dry weight. On the other hand, 5×10^{-3} M NaF visibly damaged about 20% of the tissues and hence its effect upon changes in the composition of the discs has little or no meaning in the present study. Concentrations of NaCl up to 20×10^{-3} M produced no injury and reduced ascorbic acid accumulation only about 10%.

Sodium mono-iodoacetate was compared with sodium acetate. The iodoacetate, at a concentration of 6.3×10^{-5} M, was without any effect, whereas

25×10^{-5} M solutions killed the discs. On the other hand 100×10^{-5} M sodium acetate was without effect. Similarly, 10^{-4} M potassium ethylxanthate was without effect, whereas a 10×10^{-4} M concentration of the same salt proved rather toxic (10 to 100% damage to the discs). KNO_3 at these concentrations has little or no effect. *p*-Nitrophenol likewise failed to give a differential response. A concentration of 5×10^{-5} M was without effect, whereas 25×10^{-5} M visibly injured from 5 to 100% of the tissue.

These four substances are obviously very toxic to leaf tissue at relatively low concentrations. Furthermore, the degree of their toxicity under the conditions used changed so rapidly with concentration that their usefulness in these studies is limited. On the other hand, the toxicity of 8-hydroxyquinoline seems to change less rapidly with concentration. Some results are summarized in table IX. The visible injury to the discs at a

TABLE IX

RESULTS OBTAINED BY INFILTRATING TURNIP-LEAF DISCS WITH SOLUTIONS OF 8-HYDROXYQUINOLINE

Expt. No.	Conc. hydroxyquinoline	Ascorbic acid accumulation		Dry weight increase	
		μg/disc	% of control	mg/disc	% of control
	M × 10^4				
6-5c	0.0	54	100	6.6	100
	0.625	46	85	5.5	83
	1.0	39	72	5.5	83
	5.0	.....	Toxic	.....	
6-5b	0.0	44	100	5.9	100
	1.0	24	55	4.0	68
	5.0	.....	Toxic	.....	

concentration of 10^{-4} M was 2%, or less, yet at this concentration both ascorbic acid accumulation and the increase in dry weight were inhibited somewhat in both experiments.

Discussion

These results indicate that the effects of various inorganic salts upon ascorbic acid accumulation in leaf discs can, to a certain extent, be ascribed to the effects of the individual ions. Thus, K^+ has been found to be either without effect or to have a slight stimulatory action as compared with distilled water. Other data indicate that Na^+ has a very similar effect. No difference has been observed in the response of ascorbic acid accumulation to these two cations. When Ca^{++} is supplied as single salts (NO_3^- or Cl^-) it appears to depress ascorbic acid accumulation, but in the presence of "balanced anions" it had a stimulatory effect. Mg^{++} and NH_4^+ have always shown a depressing action, although the former has been studied only in the presence of "balanced anions." Similar generalizations can be made for the anions which have been studied. Nitrate ions may have little or no effect

or may stimulate ascorbic acid accumulation. Sulphate and chloride ions apparently have either no effect, or a depressing action. On the other hand, $H_2PO_4^-$ has a marked depressing action, at least in the presence of "balanced cations." In the presence of ammonium ions, $HPO_4^=$ had about the same effect as Cl^- and $SO_4^=$. The pH, of course, influences the relative concentration of the phosphate ion species present. The "balanced cation" solutions were pH 4.3; the ammonium phosphate solution was pH 7.4. Thus the difference in response between these solutions may have resulted from differences in the relative amounts of the different phosphate ion species.

The effects of ions upon ascorbic acid accumulation can be interpreted in part, at least, in terms of a cation–anion balance in much the same manner as have similar effects of various ions upon the organic acid content of other plant tissues (**4, 6, 15, 16, 19, 24, 25**). Compare, for example, Cl^- and NO_3^-. The nitrate can be metabolized leaving an excess of cations. The tendency will be for organic anions (acids) to be formed to balance the cations. Apparently ascorbic acid contributes to the organic anions formed although its contribution may be small due to its relatively low concentration. In a comparison between K^+ and NH_4^+ a similar situation occurs, except that in this case organic anions must be reduced in amount to balance the NH_4^+ metabolized by the cells. The relative effects of ammonium and nitrate ions upon ascorbic acid accumulation are likewise similar to those observed with other organic acids in higher plant tissues (**5, 17, 27**). Ammonium ions result in smaller amounts of the various organic acids in the tissues than do nitrates. A similar situation has been observed for ascorbic acid in the leaves of pineapple plants (**21**). Furthermore, a close correlation was observed between the total organic acid and ascorbic acid contents of pineapples by HAMNER and NIGHTINGALE (**7**), but such a correlation does not apply to the diurnal fluctuation in organic acid content of the leaves of the same plant (**22**).

The overall influence of various ions is complicated, of course, by the different rates at which they enter the cells, but it appears probable that ascorbic acid is contributing to the system that tends to maintain a cation–anion balance in plant tissues, and its changes in response to various salts can be interpreted on this basis. Essentially the same interpretation was made by MAPSON *et al.* (**13, 14**) of the influence of various salts upon the ascorbic acid content of cress seedlings. These workers observed differences in the pH of the tissues as a result of supplying different salts to the seedlings (**13**). They also found that sodium salts of various organic acids stimulated ascorbic acid accumulation. The results described above indicate little or no influence of inorganic salts upon the pH of the tissues, and organic acids (at pH 5) were found either to have little or no effect upon ascorbic acid accumulation, or to depress it. Malate and succinate depressed ascorbic acid accumulation particularly at the higher concentrations (0.02 M). Mapson *et al.* observed a stimulation of ascorbic acid accumulation with the sodium salts of these acids, but little or no effect with the free acids.

Tartrate and, especially, malonate depressed ascorbic acid accumulation markedly in the present experiments. It is probable that these two acids would be metabolized less readily than succinate or malate and their effect may be simply the result of supplying relatively large amounts of non-metabolized anions to the cells. However, their effects may be more specific, particularly since they reduce ascorbic acid accumulation much more than does NaCl at the same concentration. The chloride ion also is thought to be essentially unchanged in the cell.

Malonate is considered to be a specific inhibitor of succinic dehydrogenase, hence the result obtained with this acid may indicate that Krebs' tricarboxylic acid cycle is concerned in ascorbic acid and dry-matter accumulation. Some data have been presented which suggest that such a cycle occurs in plant tissues. Stauffer has presented a summary of these data and concludes that the evidence is consistent with such a hypothesis, but he points out that direct evidence for the occurrence of a tricarboxylic acid cycle in higher plants is lacking (**23**). More recent evidence which supports the hypothesis that such a cycle occurs in plant tissues has been provided by LATIES (**11, 12**), PUCHER and VICKERY (**18**), and VICKERY and ABRAHAMS (**26**). Tartrate may form complexes with a metal required for the metabolism of the cells, *e.g.*, copper, and this may explain its action. The influence of 8-hydroxyquinoline suggests that heavy metals, possibly copper, are concerned in the processes studied in these experiments.

The relatively great toxicity of acetate and citrate is rather puzzling. Possibly citrate is forming a complex with one or more metals required for normal cell metabolism, but this seems unlikely since the concentration of citrate supplied is not of a greatly different order of magnitude from that found in other non-succulent plant tissues.

It is significant that ascorbic acid accumulation has not been studied as an isolated process. In the studies by MAPSON *et al.* (**13, 14**) growth and, in some cases, photosynthesis was occurring simultaneously, and these workers have not described in detail the influence of their treatments upon these processes in cress seedlings. In the present studies photosynthesis was a concomitant process with ascorbic acid accumulation. In other studies photosynthesis, growth, or other processes have occurred simultaneously with the changes in ascorbic acid. This complicates an interpretation of the results obtained. It is interesting to note that a rather high correlation has been obtained between the accumulation of dry matter and ascorbic acid in leaf discs. Other workers have observed a similar correlation with intact plants (**3, 9, 10**). This suggests that these two processes are intimately associated in some manner. Earlier workers have speculated about such an association (see ÅBERG, **1**). It might be suggested that photosynthesis merely provides sugars which are then converted into ascorbic acid. ÅBERG (**2**) has discussed such a possibility. MAPSON *et al.* (**14**) have suggested that hexoses may be the precursors of ascorbic acid. ÅBERG (**2**) stimulated ascorbic acid accumulation in excised tomato leaves in the dark by feeding them sucrose, but with leaves of other plants the ascorbic acid

did not increase in the dark when they were fed sucrose. However, the rate at which the ascorbic acid was broken down was retarded by this sugar. Various attempts in our laboratory to influence ascorbic acid accumulation in illuminated leaf discs by infiltration with sucrose, glucose, or L-sorbose have failed. Moreover, the ascorbic acid accumulation in discs from excised leaves starved in the dark until they no longer contain starch can be as rapid under otherwise comparable conditions as that in discs from leaves immediately after they are removed from the plant and contain abundant starch (Somers and Kelly, unpublished). This may simply mean that, in these experiments, carbohydrates were not limiting ascorbic acid accumulation and does not rule out the possibility of a rather direct synthesis of ascorbic acid from such compounds. However, if photosynthesis is merely supplying carbohydrates from which ascorbic acid is formed it seems that ascorbic acid accumulation should have been influenced by these treatments.

There is considerable evidence in the above data that, while ascorbic acid accumulation is closely associated with changes in the amount of dry matter, it responds differently in various respects. The regression between the two processes, as influenced by inorganic salts, suggests that appreciable photosynthesis may occur without any change in ascorbic acid content of the tissues. Moreover, ascorbic acid accumulation is influenced somewhat more than net photosynthesis as a result of infiltration with various salts. The accumulation of dry matter is influenced by the various inorganic salts, but on a relative basis ascorbic acid accumulation is influenced somewhat more. This is illustrated rather well by the influence of $H_2PO_4^-$ in the presence of balanced cations. With 0.005 M $H_2PO_4^-$ ascorbic acid accumulation and the increase in dry weight were 14 and 59%, respectively, of that obtained with distilled water. With 0.01 M $H_2PO_4^-$ the corresponding values were −77 and 43%, respectively. In a similar way malonate and tartrate influenced ascorbic acid accumulation relatively much more than they did the increase in dry matter. Other cases of a similar nature could be cited.

On the other hand, 8-hydroxyquinoline, which essentially was not ionized at the pH used, influenced ascorbic acid and dry-matter accumulation to about the same extent. This indicates that there are one or more reactions in common in the two processes and that copper or some other heavy metal is involved in this reaction (or reactions). This may explain the correlation between ascorbic acid accumulation and net photosynthesis. This does not necessarily imply, however, that photosynthesis is simply forming sugars which then are converted to ascorbic acid; indeed, some of the observations cited above suggest otherwise. The fact that the two processes differ in their sensitivity to reagents which would tend to change the pH of the cell suggests an indirect relationship. Ascorbic acid appears to form a part of the system in the cells which tends to maintain a balance between anions and cations. The final products of photosynthesis probably are not involved directly in such a system.

Summary

1. It has been demonstrated that infiltration with solutions of various inorganic salts will influence ascorbic acid accumulation in illuminated leaf discs of turnips and broccoli. Ascorbic acid accumulation is depressed by ammonium salts in general, but the magnitude of this effect is determined by the anion present. For example, chlorides and sulphates depress ascorbic acid accumulation more than nitrates. This relationship between these two anions applies also to calcium and potassium salts. The nature of the cations also influences the magnitude of ascorbic acid accumulation—potassium, calcium, and ammonium ions represent a series in which potassium ions have about the same influence as distilled water, ammonium ions reduce the rate of accumulation rather greatly, and calcium ions effect an intermediate response. If the salts are supplied in solutions which are more or less physiologically balanced similar relationships between the various ions are observed. When $H_2PO_4^-$ is supplied as the single anion source in the presence of a balance of Ca^{++}, Mg^{++}, K^+, and NH_4^+, it causes a very marked decrease in the rate of ascorbic acid accumulation.

2. Infiltration with salts of various organic acids (pH 5.0) also influences ascorbic acid accumulation. Citrate and acetate depress this process markedly at relatively low concentrations (0.005 to 0.01 M). Malonate and tartrate also depress ascorbic acid accumulation, whereas succinate and L-malate have little or no effect at similar concentrations.

3. 8-Hydroxyquinoline reduces both ascorbic acid accumulation and net photosynthesis, each to about the same extent. Most of the salts, both inorganic and organic, influence ascorbic acid accumulation somewhat more than the accumulation of dry matter, if they have any effect at all. Nevertheless, a close correlation has been observed between the changes in ascorbic acid and dry matter. With the inorganic salts a correlation of $+0.83$ ($n = 156$) was observed between these two variables.

4. In the discussion of these results it is suggested that ascorbic acid is contributing to the cation–anion balance in leaf tissue cells. However, this contribution may be small due to the relatively low concentration of this acid. Nevertheless, the cation–anion balance may be a factor in determining the amount of ascorbic acid accumulated. Various other interpretations of the data are discussed.

The authors wish to express their appreciation to the following for technical assistance in this work: Jeanne Darlington, Esther Hollister, Maurice V. Marion, and Floyd Morter.

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THE RESPONSE OF YOUNG VALENCIA ORANGE TREES TO DIFFERENTIAL BORON SUPPLY IN SAND CULTURE

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Received September 26, 1950

Previous reports on the boron nutrition of citrus have been concerned chiefly with deficiency and toxicity responses (**2, 3, 5, 6, 8, 9**). The objective of the present study was to maintain trees at different levels of boron between these two extremes and to observe any differences that occurred in regard to general growth pattern, mineral composition of the leaves, and fruiting behavior.

Materials and methods

Twelve young Valencia orange trees which were budded on Rough lemon stock, were planted into 50-gallon containers on April 28, 1947. The containers were perforated in the bottom and filled with a very fine, white quartz sand. These units were depressed in soil for nearly their full length. The capillary contact with the subsoil induced good drainage and permitted good root development throughout the container. A complete nutrient solution, described by HOAGLAND and ARNON (**5**), was applied twice weekly for the first month. By May 28 the trees all showed new growth and were assigned treatments in four blocks of three trees each. The rate of boron used in the nutrient feeding was the only differential variable for the succeeding three years. The lowest boron level was that which was supplied as impurities in the C.P. salts and the lake water used as a water source. During the second year of growth obvious boron deficiency symptoms (**9**) appeared at this lowest level and boron was thereafter applied at the rate of 0.01 p.p.m. A medium boron level of 0.5 p.p.m. and a high of 2.0 p.p.m. were maintained as the other two treatments.

After 18 months the basal nutrient solution was modified as follows: P lowered from 31 to 10 p.p.m.; K from 235 to 180 p.p.m.; Mg increased from 47 to 72 p.p.m.; and the micro-nutrients, Mn, Zn, and Cu increased five-fold over the relatively low levels recommended for the Hoagland solution.

Solution was applied twice weekly throughout the period of study at the rate of 2 liters per application for the first year, 2½ liters for the second year, and 3 liters during the third. Water was applied between nutrient feedings in amounts that induced leaching.

Leaf samples were collected each year and analyzed for various major elements, as previously described (**11**). The mico-nutrient elements were determined by A.O.A.C. methods (**1**), with the exception of Fe, which was determined spectrographically.

Trunk diameter measurements were made semi-annually. Fruit was allowed to develop during the third year and was analyzed for total soluble solids, ascorbic acid, and citric acid.

Results and discussion

In general, excellent growth was made by all trees. The size attained was equal to or greater than identical trees growing in soil adjacent to the plots. All growth was nearly normal in appearance except that the low-boron trees showed mild deficiency symptoms in the foliage (**9**) during the fall months of the second year, and the high-boron trees showed mild toxicity symptoms of occasional tip burn and yellow spots (**2**) throughout the test period. These symptoms were more pronounced during 1948, when the mean total boron content in dry leaf samples was 386 p.p.m., than in 1949 and 1950 when it was about 265. The high-boron trees showed some tendency toward forming a less compact top than the others. They had fewer, but larger, branches and a more open character. The mean tree size was nearly identical in all three treatments, as is indicated by the cross-sectional trunk area measurements in table I.

The leaf samples collected on the first three and last sampling dates shown in table I were mature spring-flush leaves. A broad range in the boron concentration within the leaf was induced and maintained. This range was over 24-fold in the summer of 1948 and over 10-fold in the summers of 1949 and 1950. The differences in the other elements in these mature samples are relatively small. Phosphorus tends to be present in slightly greater concentrations when boron is low. Such a relationship has previously been found with sunflowers (**6**).

Three samplings were made from young leaves which were developing in the fall at the time that a crop of fruit was maturing. Under these conditions the difference in the P concentration was greater than with mature leaves, although the difference appeared to diminish as the leaf approached maturity. The concentrations of the three base elements, K, Ca, and Mg were also influenced in these younger leaves. When boron was supplied in a very limited amount Mg had a tendency to enter the leaf in greater amounts, and reciprocally, K in lesser amounts. Calcium appears to have been depressed at the highest boron level. Here again it appears that these differences are perhaps temporary and tend to diminish as the leaf grows older.

Nitrogen, manganese, copper, iron, and zinc do not appear to have been influenced in any way by the variation in boron supply. Sodium was determined on the same collections for which iron values are shown and showed no differences which were attributable to the rate of boron supply.

From 10 to 15 pounds of oranges were produced by each tree during 1949. These were picked and analyzed on February 6, 1950. No systematic differences were found in the yield, fruit size, rind thickness, juice content, or percentage of total soluble solids and citric acid in the juice. The

TABLE I

THE RELATION OF BORON LEVEL TO CROSS-SECTIONAL AREA OF THE TRUNK, AND DRY WEIGHT AND MINERAL COMPOSITION OF LEAVES OF YOUNG VALENCIA ORANGE TREES AT DIFFERENT INTERVALS OF A THREE-YEAR CULTURE PERIOD. TREES PLANTED APRIL 28, 1947.

Sampling date and leaf age	Boron applied (p.p.m.)	Mean trunk X-section ($cm.^2$)	Mean leaf weight (mg.)	Percentage of leaf dry matter					P.p.m. in leaf dry matter				
				N	P	K	Ca	Mg	B	Mn	Cu	Zn	Fe
9-4-47	0.00	3.08	193	2.82	0.150	1.84	2.95	0.395	41	42	8	...	...
5 months	0.50	3.07	176	2.78	0.171	1.77	2.98	0.360	90	47	9	...	...
	2.00	2.73	193	2.71	0.136	1.79	2.98	0.352	117	41	8	...	...
7-10-48	0.00	4.89	274	2.91	0.164	2.86	2.42	0.194	16	33	8	...	74
6 months	0.50	4.75	272	2.89	0.156	2.84	2.55	0.194	144	44	9	...	80
	2.00	4.49	330	2.81	0.161	2.92	2.52	0.202	386	46	8	...	64
8-5-49	0.01	14.56	364	2.26	0.133*	1.84	2.68	0.288	25	31	14	47	83
6 months	0.50	14.77	358	2.25	0.121	1.72	2.84	0.242*	93	34	14	41	83
	2.00	13.89	389	2.15	0.112	1.99	2.70	0.267	263	30	13	43	76
8-30-49	0.01	...	414	2.15	0.178**	1.84**	2.61	0.512**	17	24	14	29	66
1 month	0.50	...	365	2.23	0.147	2.13	2.69	0.442	58	35	14	24	68
	2.00	...	379	2.12	0.146	2.15	2.20**	0.444	130	25	14	25	65
9-29-49	0.01	...	458	2.44	0.175**	1.82**	2.90	0.524**	20	28	13	29	...
2 months	0.50	...	419	2.38	0.140	2.04	2.74	0.435	67	32	15	36	...
	2.00	...	431	2.34	0.137	2.25	2.50**	0.421	158	29	14	36	...
12-5-49	0.01	18.13	506	2.30	0.157*	1.65	2.98	0.450*	24	44	13	29	...
4 months	0.50	18.18	454	2.35	0.133	1.68	2.98	0.400	78	45	14	28	...
	2.00	18.36	477	2.31	0.138	2.03**	2.55**	0.398	168	36	13	33	...
6-8-50	0.01	20.52	304	2.35	0.119	1.48**	2.46	0.349	25	43	12	34	...
5 months	0.50	20.67	314	2.20	0.117	1.80	2.54	0.312	104	38	12	33	...
	2.00	20.87	326	2.27	0.114	1.78	2.41	0.351	262	40	11	32	...
L.S.D. between any two means	@ 0.05	N.S.	N.S.	N.S.	0.020	0.22	0.27	0.040	2.7	N.S.	N.S.	N.S.	N.S.
	@ 0.01	...	...	...	0.027	0.30	0.36	0.053	3.6	...	...	...	...

*Signifies that the odds for significance exceed 19:1.
**Signifies that the odds for significance exceed 99:1.

only difference that was consistent in all four replications was a reduction in the ascorbic acid content of the juice in the low-boron trees. This treatment averaged 49.8 mg. per 100 ml., as against 57.0 and 54.9 for the medium- and high-boron treatments, respectively, a response which may be indirectly attributable to the boron supply, however, and more closely associated with the higher level of phosphorus in the low-boron trees. The last relationship was found to exist under orchard conditions when the leaf phosphorus was increased without changing the boron status of the trees (**10**).

The literature on boron nutrition shows several instances with various plants of a lack of growth response to a differential supply of this element between the limits of deficiency and toxicity levels. The present limited study with Valencia oranges seems to provide no exception to that rule. Apparently normal trees can be grown with very limited applications of boron if this element is supplied at frequent intervals. Likewise, applications of boron in amounts which produce mild toxicity symptoms do not seem to interfere appreciably with the functioning of the plant. The evidence presented is the first to show the relatively small effect of rather large variations in the boron content (maximum range 16 to 386 p.p.m.) of citrus on growth, fruiting, and the concentration of other mineral elements in the leaves. A similar range (30 to 305 p.p.m. boron) in mature Valencia orange leaves was found in a recent survey (**7**) of 75 commercial orchards in the major citrus-producing areas of the United States.

Although the critical levels of leaf boron associated with either deficiency or toxicity symptoms are undoubtedly conditioned by several factors, it is of interest to note that the values obtained here are in essential agreement with previous work. Deficiency was associated with somewhat less than 20 p.p.m. in mature leaves. Toxicity began somewhere near 265 p.p.m., at which point only the slightest tip burning occurred. Respective critical values of 15 and 200 p.p.m. are suggested by California workers (**3**).

Summary

Young Valencia orange trees were grown for three years in large outdoor sand cultures on complete nutrient solutions that varied differentially only in the amount of boron. Three rates of boron were applied to single-tree plots. The plots were replicated four times.

No difference in tree size resulted from the differential treatments.

Rather large differences in the boron content of the leaves were induced. The low-boron plants showed mild foliage deficiency symptoms during the second year but not in the first or third years of growth. The high-boron plants showed slight leaf symptoms of toxicity throughout the three-year period.

Mature leaves showed virtually no differences in mineral composition other than the 10- to 24-fold difference in boron. Phosphorus tended to be present in slightly greater concentration in the presence of low boron.

Young leaves showed this same relationship with phosphorus in a more pronounced manner. When the rate of boron supply was low, potassium accumulation in the leaf was retarded and magnesium accumulation accentuated. The rate of calcium accumulation was depressed at the highest boron level. These differences appear to diminish as the leaf approaches maturity.

The only consistent difference in the quality of the fruit produced during the third year was a slight reduction in the ascorbic acid content in the low-boron cultures.

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EFFECTS OF DIFFERENT CONCENTRATIONS OF NITRATE ON THE GROWTH AND VITAMIN CONTENT OF OAT PLANTS[1]

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(WITH THREE FIGURES)

Received June 10, 1950

Only recently have studies been conducted concerning the effects of mineral elements upon the vitamin content of plants. These studies have dealt largely with carotene and ascorbic acid content. The effect of mineral elements upon the biosynthesis of the B-complex vitamins has received very little attention. WATSON and NOGGLE (**11**) studied the effects of mineral deficiencies upon riboflavin synthesis in immature oat plants and found a high positive correlation between nitrogen and riboflavin concentrations in the leaves. MC COY *et al.* (**7**) also using immature oat plants, studied the effects of sub-optimum concentrations of mineral elements upon the synthesis of niacin, and demonstrated that there was a decreasing concentration of niacin with decreasing concentrations of nitrogen supplied to the plants.

This paper is a report of a study of the effect of varying concentrations of nitrate nitrogen in the nutrient solution on the synthesis of biotin and pantothenic acid in immature oat plants.

Experimental procedures

The plants used in this experiment were Wintok oats (*Avena sativa* L. var. Wintok), No. CI 3424, the seed having been obtained from the Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma. The oat seeds were germinated in quartz sand which was watered with a nitrogen-deficient nutrient solution. After emergence of the coleoptile, the plants were carefully selected for uniformity and transplanted to quartz sand in glazed crocks of approximately 2-quart capacity. The sand used was "Flint Shot" obtained from the Ottawa Silica Company, Ottawa, Illinois. It was thoroughly washed with tap and distilled water before using.

The nutrient solutions used were basically those of HOAGLAND and ARNON (**5**), Solution I, Method B. Five different concentrations of nitrate varying from 2.5 to 22.5 millimoles per liter were used (table I). The osmotic pressure of each solution was adjusted to 1.07 atmospheres with sodium chloride. The pH was adjusted to 5.5 with HCl and was found to remain constant during the experiment. Sufficient iron, in the form of ferric citrate, to make a concentration of 0.5 p.p.m. was added separately with each watering. Each pot was watered with approximately 100 ml. of nutrient solution each day.

[1] This work was done at the University of Oklahoma and was supported in part by a fellowship from The Samuel Roberts Noble Foundation.

The oats were planted on February 15, 1949. The seedlings were transplanted to their respective pots on February 25, 1949. Ten pots with five plants in each were used for each of the five treatments. The plants were harvested at the end of 84 days on May 10, 1949.

Analytical procedures

The oat plants were clipped at sand level and individual fresh-weight determinations made immediately. Thus the individual fresh weight was obtained for each of the 250 plants. For the vitamin determinations, fifteen plants from each treatment were selected at random. After weighing, each of these plants was immediately homogenized in a Waring Blendor with 35 ml. of 0.5 M sodium acetate solution which had been adjusted to pH 4.5. The homogenized material from each of the plants was transferred quantitatively to a 400-ml. beaker, and to this was added 5 ml. of a freshly prepared enzyme suspension containing 20 mg. of papain and 20 mg. of takadiastase

TABLE I

COMPOSITION OF NUTRIENT SOLUTIONS IN MILLIMOLES PER LITER*

Conc. of nitrate/l.	KNO_3	$MgSO_4$	KH_2PO_4	$Ca(NO_3)_2$	$CaCl_2$	$NaNO_3$	KCL	NaCl
2.5	2.5	2.0	1.0	0.0	5.0	0.0	2.5	8.5
10.0	5.0	2.0	1.0	0.0	5.0	5.0	0.0	3.5
15.0	5.0	2.0	1.0	5.0	0.0	0.0	0.0	8.5
20.0	5.0	2.0	1.0	5.0	0.0	5.0	0.0	3.5
22.5	5.0	2.0	1.0	5.0	0.0	7.5	0.0	1.0

*Hoagland and Arnon micronutrients and ferric citrate to make 0.5 p.p.m. were added.

per ml. (1), and 5 ml. of an extract of hog kidney conjugase. The hog kidney conjugase was unnecessary for making determinations of biotin and pantothenic acid. At the time the plants were harvested, however, it was planned that folic acid determinations might be made. Folic acid is set free from its conjugated condition by hog kidney conjugase (2). Following addition of the enzymes the mixture was incubated at 37° C for 24 hours. Enzymatic action was stopped by placing the material in flowing steam in an autoclave for 30 minutes. The digested samples were filtered through Whatman #50 filter paper, diluted to a volume of 1 liter, and then stored in the dark at 4° C until the vitamin content could be determined. Under these conditions vitamin activity loss is reduced to a minimum (1).

Thirty-five plants from each treatment were used for dry-weight determinations. These were placed individually in paper sacks and dried in a forced-air drying oven at 95° C for 24 hours. After drying they were stored in desiccators until their dry weight could be determined.

For nitrogen determinations the plants from each treatment then were divided into five composite samples, each containing seven plants. Thus

for the entire five treatments there was a total of 25 samples. These samples were ground to pass a 40-mesh screen by the use of a semi-micro Wiley mill. Nitrogen was determined by a modified micro-Kjeldahl method using metallic selenium and potassium sulphate. Duplicate determinations were made on each composite sample. The means of the duplicates of the five composite samples from each treatment were averaged and this value is listed in table II as the nitrogen content of the plants from each treatment.

The methods of microbiological assay for the determination of biotin and pantothenic acid were essentially those employed by WRIGHT and SKEGGS (**12**), and SKEGGS and WRIGHT (**9**), respectively. Corrections for the amounts of pantothenic acid and biotin in the enzyme suspensions were made. Growth rate measurements were determined turbidimetrically by use of a Coleman Model II Universal Spectrophotometer at 660 mμ after 18 hours of growth. Optical densities were read directly from the instrument. This procedure results in somewhat greater accuracy than can be obtained by reading percentages of transmission.

Triplicate determinations of pantothenic acid and biotin were made for each plant and the mean value of these determinations was used in calculating the vitamin content per gram of fresh weight for each plant. The values for each plant within a single treatment were then averaged and the means appear in table II. Analysis of variance for the triplicate determinations from each plant was not made, and only the variance between plants was used in calculating the standard error.

Results and discussion

The plants that received 10, 15, 20, and 22.5 millimoles of nitrate per liter of nutrient solution, respectively, were very much alike in external appearances, and no symptoms of nitrogen deficiency were visible to the observer. All plants of these groups possessed a uniform dark green color. All plants grown in the nutrient solution containing 2.5 millimoles of nitrate per liter were stunted and chlorotic, and exhibited uniformly typical nitrogen-deficiency symptoms.

Nitrate concentration had a very pronounced effect upon the production of fresh and dry weight in immature oat plants (fig. 1). This effect has been demonstrated by several other workers (**3**, **8**, **10**). An increase from 2.5 to 10 millimoles of nitrate per liter of nutrient solution resulted in an increase in fresh-weight production of 176.5%, and in dry-weight production of 147.8%. This increase was highly significant in both cases ($P < 0.01$). The differences in fresh- and dry-weight production between those plants receiving 10, 15, 20, and 22.5 millimoles of nitrate were not significant.

When increasing concentrations of nitrate were applied to the plants there was a corresponding increase in the number of tillers per plant (fig. 2). Plants which received 22.5 millimoles of nitrate per liter of nutrient solution possessed 95% more tillers per plant than those receiving 2.5 millimoles. There was a highly significant ($P < 0.01$) increase in the number of tillers

TABLE II

COMPOSITION OF OAT PLANTS SUPPLIED WITH DIFFERENT CONCENTRATIONS OF NITRATE

Conc. of NO_3 in nutrient solution	Average fresh weight	Average dry weight	Numbers of tillers per plant	Nitrogen* per cent dry weight	Pantothenic acid per gram fresh weight	Biotin per gram fresh weight
Millimoles per liter	*grams*	*grams*		%	*γ*	*mγ*
2.5	2.32	0.34	7.36 ± 0.22**	3.48	0.99 ± 0.11**	3.14 ± 0.09**
10.0	6.43	0.85	11.12 ± 0.32	4.58	2.58 ± 0.32	8.18 ± 0.22
15.0	6.74	0.89	11.72 ± 0.23	4.99	2.15 ± 0.18	8.53 ± 0.18
20.0	7.39	0.98	12.46 ± 0.28	5.16	2.57 ± 0.18	8.34 ± 0.16
22.5	7.42	1.03	13.36 ± 0.32	5.75	2.02 ± 0.14	8.00 ± 0.27

*Kjeldahl nitrogen.
**Standard error.

per plant when the nitrate was increased from 2.5 to 10 millimoles of nitrate per liter of nutrient solution. A significant increase ($P < 0.05$) was found to occur when those plants grown in 15 millimoles of nitrate were compared with those grown in 20 millimoles. Also there was a significant increase ($P < 0.05$) when those plants grown in 20 millimoles were compared with those grown in 22.5 millimoles of nitrate per liter of nutrient solution. The difference between plants grown in 15 millimoles of nitrate per liter of nutrient solution and those in 22.5 millimoles was highly significant ($P < 0.01$).

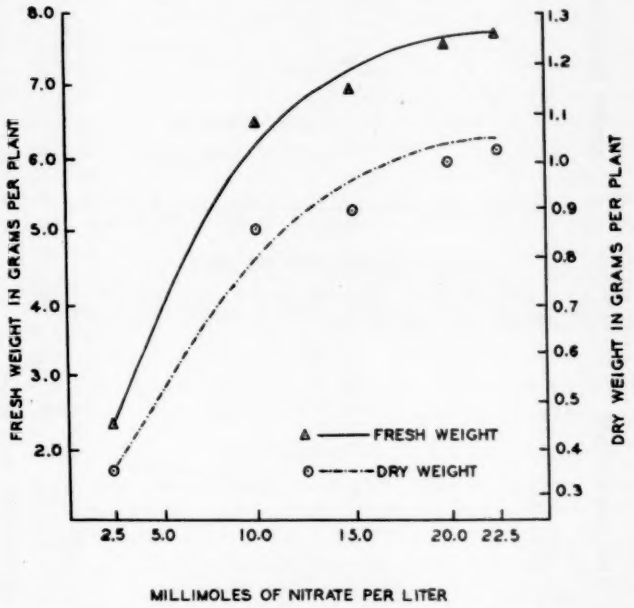

FIG. 1. Average fresh and dry weight of oat plants grown with nutrient solutions containing different concentrations of nitrate.

DICKSON (3) presented data which indicated that reduction of nitrogen produced a greater reduction of tillers than was produced by any other major elements except phosphorus.

The percentage of Kjeldahl nitrogen (dry-weight basis) roughly increases as the amount of nitrate applied in the nutrient solutions is increased (fig. 2). Those plants receiving 22.5 and 10 millimoles of nitrate per liter of nutrient solution contained 65.2 and 31.6% more nitrogen, respectively, than did the plants receiving 2.5 millimoles of nitrate.

When immature oat plants were grown under the conditions of this experiment an increase of the nitrate concentration in the nutrient solution

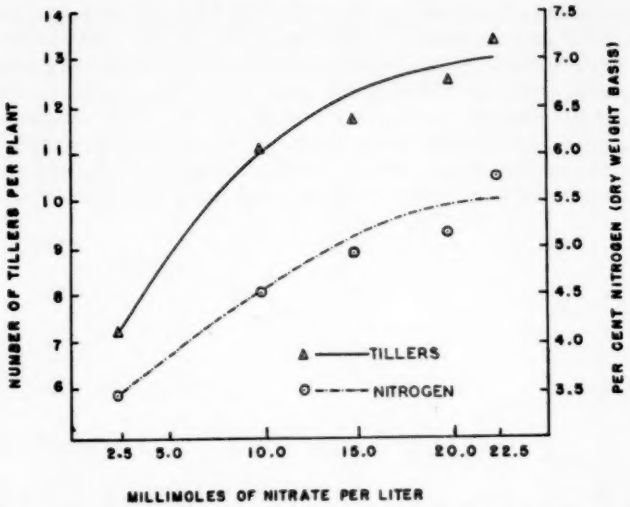

FIG. 2. Average number of tillers per plant of oat plants and nitrogen content grown with nutrient solutions containing different concentrations of nitrate.

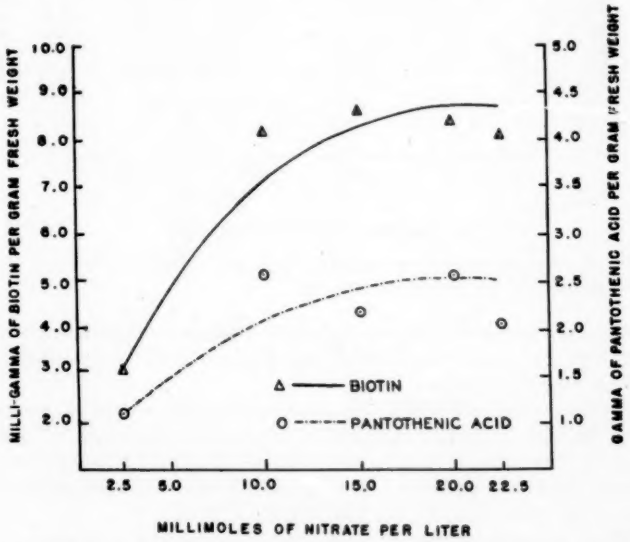
MILLI-GAMMA OF BIOTIN PER GRAM FRESH WEIGHT
GAMMA OF PANTOTHENIC ACID PER GRAM FRESH WEIGHT
BIOTIN
PANTOTHENIC ACID
MILLIMOLES OF NITRATE PER LITER

FIG. 3. Biotin and pantothenic acid content of oat plants grown with nutrient solutions containing different concentrations of nitrate.

from 2.5 to 10 millimoles per liter resulted in a highly significant ($P < 0.01$) increase of both pantothenic acid and biotin per gram of fresh plant material (fig. 3). While there were smaller amounts of both pantothenic acid and biotin in the plants which received the higher concentrations of nitrate, none of the differences found in the plants receiving more than 10.0 millimoles of nitrate were significant. These apparent decreases are of interest, however, and deserve further study. It should be noted that the chloride content of the nutrient solutions varied somewhat inversely with the concentration of nitrate. It is possible that these differences in chloride concentration may have resulted in some of the observed differences in the plants in these experiments.

It is perhaps noteworthy that WATSON and NOGGLE (**11**) and MC COY *et al.* (**7**) found lesser amounts of riboflavin and niacin content respectively, when the nitrogen in the nutrient solution was lowered to such an extent that the plants were exhibiting deficiency symptoms. The results of these studies indicate that the amount of nitrogen available to immature oat plants may produce a very marked effect upon their B-vitamin content. MOYER *et al.* (**6**) in comparing the pantothenic acid content of oats (var. Wintok) during different years found a significant increase in the pantothenic acid content when growing conditions were particularly favorable.

The values for the pantothenic acid content cited by ELVEHJEM and SOBER (**4**) for dehydrated oat grass and by MOYER *et al.* (**6**) who used the same variety of oats as used in this experiment, were of the same order as those found in this experiment in plants receiving 10, 15, 20, and 22.5 millimoles of nitrate per liter of nutrient solution.

Summary

Oat plants were grown in sand cultures with nutrient solutions containing 2.5, 10, 15, 20, and 22.5 millimoles of nitrate per liter.

Significant increases were found in fresh weight, dry weight, tillering, pantothenic acid, and biotin when plants grown in 2.5 millimoles were compared with those grown in 10.0 millimoles of nitrate per liter of nutrient solution.

Significant increases in tillering were found to occur between all treatments with the exception of those plants growing in 10 and 15 millimoles of nitrate per liter of nutrient solution.

The author wishes to express his appreciation to Dr. Lawrence M. Rohrbaugh and to members of the Plant Sciences Department of the University of Oklahoma, for their assistance and suggestions during the course of this investigation. The author is also indebted to Robert Steele, C. L. Dulaney, Paul Harris, M. K. Patterson and Lynn Grist, who assisted in harvesting and homogenizing the plant material.

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THE AMINO ACIDS OF ALFALFA AS REVEALED BY PAPER CHROMATOGRAPHY WITH SPECIAL REFERENCE TO COMPOUNDS LABELLED WITH S^{35}

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(WITH FOUR FIGURES)

Received July 25, 1950

This investigation is essentially a collaboration between two laboratories bringing together problems in which each is interested.

The Department of Agricultural Research of the American Smelting and Refining Company has long been interested in the problems of sulphur toxicity and sulphur metabolism in plants which are economically important. In the course of this work investigations have been made by the use of S^{35}. These have shown the general distribution of the isotope in the plant body. To some extent chemical procedures have been employed to identify in the protein hydrolysate specific sulphur-containing compounds, notably cystine and probably methionine (**8, 9,** and **10**). It remained, however, necessary to obtain unequivocable evidence on the sulphur compounds into which the isotope had entered.

In the Department of Botany at Rochester, investigations have been in progress since 1946 utilizing the techniques of paper chromatography to identify the free and combined amino acids in plants (**2, 6,** and **7**). The sulphur-containing amino acids present rather special problems. Due to the small quantities commonly present and the poor reactivity of these substances with ninhydrin, they tend to be less easily detectable by the methods of paper chromatography than many of the other amino acids (**1**). Furthermore, as two-directional paper chromatograms are commonly carried out (*i.e.*, using phenol : collidine-lutidine), the sulphur-containing amino acids tend to be obscured by others which are commonly present. Methionine sulphoxide occupies the same area as γ-amino butyric acid, a newly discovered free amino acid which is widely distributed (**4, 6,** and **7**). Methionine itself is close to the leucines, and cystine can best be recognized if it is first converted to cysteic acid which occupies a position on the chromatograms well apart from other amino acids (**1**). In these circumstances the use of S^{35} combined with autoradiographs of paper chromatograms is an obvious device to aid in the identification of the sulphur-containing amino acids.

In the laboratories of the American Smelting and Refining Company alfalfa plants were treated with S^{35} in the form of sulphate. The alfalfa

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plants were grown in sand; prior to the treatment with $S^{35}O_4^=$ the shoots were removed, then the radioactive substance was added to the nutrient solution and the new growth which developed was examined. New leaves, formed after the addition of $S^{35}O_4^=$, contained much more (25 times) S^{35} than similar leaves which were already formed. Leaf extracts of various types were prepared and sent to Rochester for further examination by paper chromatographic methods.

Two fractions that were of greatest interest were the 80% alcohol extract and the alcohol-insoluble residue: the former contains the free amino acids while the latter contains the proteins. Of secondary interest was the expressed juice from frozen leaves from which the protein was removed by heat coagulation; the liquid portion is comparable to the alcohol extract.

The heat coagulum contains the globulins and albumins of the cells. The residue from pressed leaves, after washing with water, alcohol and 0.05 *N* HCl, should include the insoluble proteins which are more structurally than metabolically important. The heat coagulum together with the water, alcohol, and acid-insoluble fraction is approximately equivalent to the alcohol-insoluble fraction mentioned above since the acid- and alcohol-soluble protein was found to be negligible.

Preparation of the extracts

The free amino acids of fresh alfalfa leaves from $S^{35}O_4^=$-treated plants were extracted with 80% ethanol by grinding in a Waring Blendor. The mixture was filtered and the filtrate evaporated *in vacuo* in a desiccator at room temperature.

The alcohol-insoluble residue was suspended in water and hydrolyzed by heating with 8 *N* HCl for 20 hours. The HCl was removed and the solution concentrated *in vacuo*.

Leaves from comparable plants were frozen at $-20°$ C and clear juice was expressed from them at 15,000 pounds per square inch. The juice was heated for a few minutes in a water bath and the heat coagulum was separated in the centrifuge. The coagulum was hydrolyzed and the hydrolysate concentrated in the manner described above. The aqueous solution, free from the coagulum, was evaporated at room temperature.

The pressed residue, washed as indicated, was also hydrolyzed and the hydrolysate concentrated *in vacuo*.

Chromatography and autoradiography

These extracts were submitted to two-directional paper chromatography.

In all cases the extract was treated at the point of application on the paper with a 30% solution of hydrogen peroxide and dried. The purpose of the oxidation was to convert methionine and its sulphoxide to the sulphone and cystine to cysteic acid. Though DENT (**4**) stated that usually no molybdate is necessary to catalyze the oxidation of sulphur-containing amino acids if 30% hydrogen peroxide is used, he subsequently corrected

this. Apparently the stabilizer in the hydrogen peroxide affects the efficiency of the oxidation so that the sulphoxide may not be completely oxidized. However, in the use of this method during this work, it was found that these oxidations did not always proceed quantitatively so that the relative amounts of the oxidized and unoxidized products varied from chromatogram to chromatogram. It is now known that this may be remedied by use of molybdate catalyst and repeated (*e.g.*, three times) treatments with hydrogen peroxide on the paper. However, for purposes of interpretation, activity due to methionine sulphone and sulphoxide may be combined and referred to as methionine derivatives and activity present both as cystine and cysteic acid may be combined as total cystine (cf. table I).

In some cases, the initial activity of the preparation was determined by placing a thin-window Geiger counter over the point of application. Subsequently, when the chromatogram was developed, the distribution of the S^{35} could be determined by counting similarly the activity of various areas of the paper.

The chromatography was carried out by the general procedure used in this laboratory (**6**). (The samples were chromatographed with neutralized phenol saturated with water followed by chromatography at right angles with 3:1 lutidine-collidine saturated with water. No ammonia, diethyl amine or hydrogen cyanide vapors were used in the cabinets.) Any residual acidity in the sample was neutralized with ammonia. The final location of the amino acids was revealed by spraying with 0.1 to 0.2% ninhydrin in alcohol and heating the papers, after spraying, at 60° C. The qualitative identification of the amino acids was made drawing upon the general experience from other work in which the pure amino acids were chromatographed. Autoradiographs could be prepared after the papers were sprayed with ninhydrin. To do this, 14″ × 17″ Eastman No-Screen X-ray film and the chromatogram were brought into direct contact and the exposures made in a refrigerator. Tests have been made by the use of a thin cellophane barrier to verify that the blackening of the film attributed to S^{35} was not due to chemical fogging (**13**). The exposures varied with the activity of the preparation from a few days to several months. After developing the film, the positions of spots due to radioactive sulphur could be compared with the spots brought out by ninhydrin. The quantity of material required for good amino acid chromatograms using ninhydrin is of the order of 50 μgm. of amino nitrogen. With the samples used, this amount of material gave adequate autoradiographs due to S^{35}.

The soluble (free) amino acids

The distribution of alcohol-soluble amino acids on the chromatogram is shown in figure 1 A and the spots are identified in figure 1 B. The noteworthy features are as follows.

Valine, the leucines, γ-amino butyric acid, and tyrosine were all present in smaller amounts, relative to other amino acids, than in many other

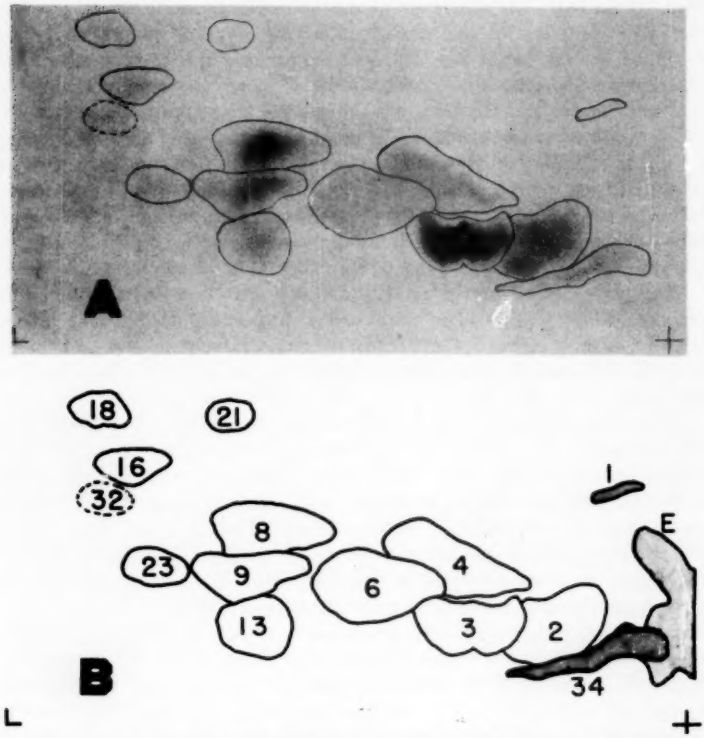


FIG. 1. Amino and S^{35} compounds in the alcohol-soluble fraction of alfalfa leaf. A. Two-directional paper chromatogram. B. Key to ninhydrin reactive and radioactive areas.

Legend: 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, serine; 6, asparagine; 8, alanine; 9, glutamine; 13, arginine; 16, valine; 18, leucine and isoleucine; 21, tyrosine; 23, γ-amino-butyric acid; 32, unknown; 34, glutathione; E, unknown, probably inorganic sulphur salts.

In this and in all subsequent figures the point of origin is in the lower right-hand corner (+) and the directions of movement are horizontal in phenol and vertical in collidine-lutidine. The following conventions have been adopted in the keys. All areas of activity, whether due to ninhydrin or S^{35}, have been outlined, a dashed outline indicating a spot of very low ninhydrin density. Areas showing ninhydrin reactivity only are plain, those showing both ninhydrin reactivity and radioactivity are striped vertically, and those showing radioactivity only are stippled. All compounds reacting with ninhydrin are designated with numbers corresponding to those used by DENT, STEPKA, and STEWARD (2). Compounds containing S^{35} but not reacting with ninhydrin are designated by letters.

common tissues that we have examined (**6**). The obvious presence of aspartic and glutamic acids, serine, asparagine, glutamine, and alanine was an expected and a common feature. The extensive ninhydrin reactive area (No. 34) near the point of origin was in the region in which glutathione is to be expected and added glutathione was found to superimpose upon this area. The presence of glutathione was confirmed by hydrolysis of the fraction since the spot disappeared and the amount of cysteic acid increased. β-Alanine, obscured by glutamine (No. 9 in fig. 1 B), was also revealed upon hydrolysis. The basic amino acids were represented only by arginine. Histidine, even if present, could not have been readily detected on these chromatograms since there was no ammonia in the cabinets. At least one unknown amino compound existed and occupied the position indicated by No. 32 and there was also a spot in the position known to be occupied by cysteic acid.

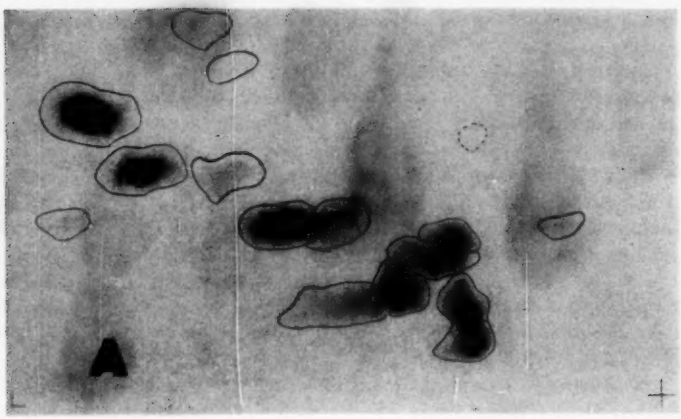
It will be noted that there was no evidence by ninhydrin of methionine sulphone, which usually occurs between valine and alanine, though it is not possible to eliminate methionine and methionine sulphoxide on the basis of the ninhydrin chromatogram alone as these might be obscured, as described above.

The autoradiograph showed radioactivity in the areas indicated on the key (fig. 1 B). It will be noted that methionine and its derivatives were either completely absent or present only in such small amounts that they were not detectable either by ninhydrin or by the radioactivity of S^{35}. The conspicuously radioactive components of the alcohol-soluble fraction appeared on the chromatogram in the area in which glutathione may be expected, with some evidence of cysteic acid derived from cystine. Inorganic forms of sulphur would not be expected to move far from the point of origin and so may have contributed to the conspicuous area shown (E). Hydrolysis of the alcohol-soluble fraction followed by chromatography and autoradiography furnished no evidence which was inconsistent with the presence of glutathione and cystine as the only S^{35}-containing organic substances in this fraction.

Examination of a water extract of alfalfa leaves, freed from heat-coagulable protein, gave results similar to those obtained in the alcohol extract. Valine, the leucines, tyrosine were all present but in relatively small amounts and the amino acids which appeared in the alcohol extract were also present in the water extract with the exception of arginine which was not detected.

Composition of protein hydrolysates

The amino acids obtained on hydrolysis of the alcohol-insoluble fraction include those to be expected from protein hydrolysis (see figs. 2 A and B). Noteworthy features, however, are the presence of unknown ninhydrin reactive substances in the positions 31A and 33: these showed no evidence of radioactivity. No. 31A is presumably identical with No. 31 in figure 3.



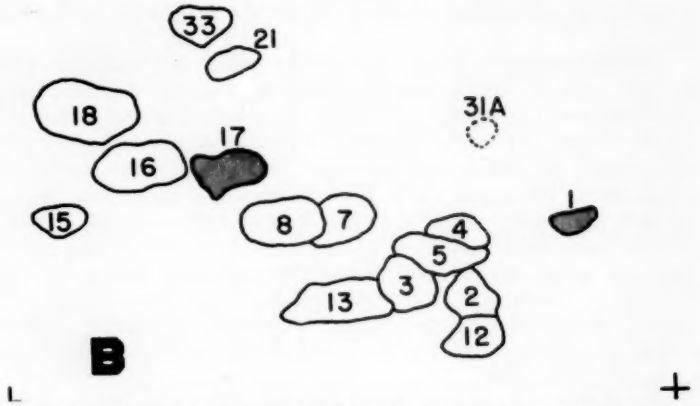


FIG. 2. Amino and S^{35} compounds in the hydrolysate of the alcohol-insoluble fraction of alfalfa leaf. A. Two-directional paper chromatogram. B. Key to ninhydrin reactive and radioactive areas.

Legend: 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, serine; 5, glycine; 7, threonine; 8, alanine; 12, lysine; 13, arginine; 15, proline; 16, valine; 17, methionine sulphone; 18, leucine and isoleucine; 21, tyrosine; 31A, unknown; 33, unknown.

The identity of 12 as a distinct spot from 2 was confirmed by the addition of lysine to the extract followed by chromatography. Thirteen was similarly confirmed. The slow movement of both lysine and arginine in the phenol direction in all these chromatograms may be attributed to the fact that chromatography was carried out in the absence of ammonia in the cabinet.

No. 33 appears to be chromatographically identical with a substance observed by R. M. Zacharius (working with Steward and Thompson) in hydrolysates of an alcohol-insoluble fraction from carrot.

In this protein hydrolysate of alfalfa leaves, valine, the leucines, phenylalanine (not shown in fig. 2 A and B, but identified in a chromatogram of a larger amount of the fraction), and tyrosine were prominent. This stands in marked contrast to the result on the alcohol-soluble fraction. Also, γ-amino-butyric acid, which was present in small amount in the soluble fraction, did not occur in the products of acid hydrolysis of the insoluble fraction. This is in accord with our general experience with this substance (6 and 7).

The basic amino acids were present in relatively large amounts in the protein hydrolysate. Lysine was easily detectable in the hydrolysate, though it did not occur free; arginine, on the other hand, was present both in the free and the combined state.

Methionine sulphone from methionine, which did not occur in quantity in the free state, appeared definitely in the protein hydrolysate and cysteic acid from cystine was also much more prominent. The autoradiograph confirms these two compounds as shown by figure 2 B, Nos. 17 and 1.

The only other S^{35}-containing compound to be found in the hydrolysate was observed in a chromatogram of four times the amount of material used in the preparation of figure 2. This was a ninhydrin nonreactive compound occurring at a position between and above serine (No. 4) and threonine (No. 7). This substance may be identical with the spot at C, figure 4, to be considered below.

Alfalfa leaves were fractionated in ways described above, yielding the following distinctive fractions:

(I) The heat-coagulable, water-soluble protein.
(II) A water-, alcohol-, and dilute-acid-insoluble fraction.

Fraction (I) was a small one, accounting for only about 2.6% of the total N. Fraction (II) was a relatively large one, accounting for about 70% of the total.

Tracings from autoradiographs and paper chromatograms of these are illustrated in figures 3 (Fraction I) and 4 (Fraction II). As in the case of figure 2, the amino acids expected to arise from protein hydrolysis appeared on the chromatograms. Three hitherto undetected ninhydrin-reacting substances appeared in the positions marked 29, 30, and 31 in figure 3. None of these unknown substances, however, contained sulphur as determined by radioactivity. No. 29 may be due to lysine since lysine hydrochloride occasionally gives two spots in positions similar to Nos. 12 and 29. Presumably No. 29 is due to the free acid and No. 12 is due to its salts. The light blue color and the shape of spot No. 30 were distinct from that of the other spots on the chromatogram, making No. 30 in all probability something other than an amino acid, possibly an ammonium salt of some other

type of acid. It should also be noted that hydroxyproline occurred in the hydrolysate of Fraction (II) (see fig. 4) although it was absent from Fraction (I) (see fig. 3). Since there is no evidence for γ-amino butyric acid in the alcohol-insoluble hydrolysate (fig. 2), all the ninhydrin reactivity at No. 14, figures 3 and 4, is ascribed to methionine sulphoxide. (The hydrogen peroxide treatment without added catalyst was not sufficient for complete oxidation of the methionine.)

The S^{35}-containing compounds which arose from hydrolysis of the two protein fractions mentioned were as follows. Methionine sulphone and sulphoxide were conspicuous (Nos. 17 and 14 of figs. 3 and 4). Cysteic acid from cystine was also definite (No. 1 of figs. 3 and 4). An unknown,

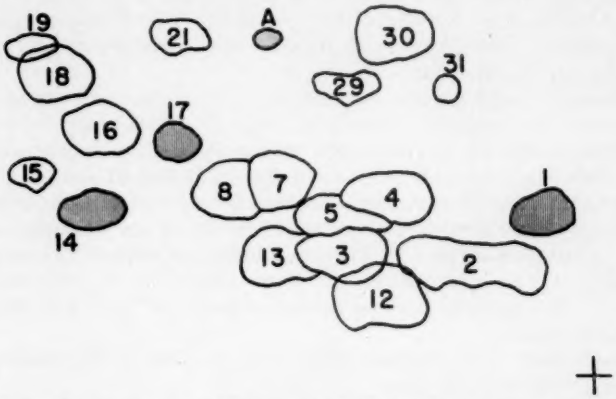


FIG. 3. Key to amino and S^{35} compounds in the hydrolysate of the coagulum from the water-soluble fraction of alfalfa leaf. Legend: 1, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, serine; 5, glycine; 7, threonine; 8, alanine; 12, lysine; 13, arginine; 14, methionine sulfoxide; 15, proline; 16, valine; 17, methionine sulfone; 18, leucine and isoleucine; 19, phenylalanine; 21, tyrosine; 28, hydroxyproline; 29, unknown, probably due to lysine; 30, unknown, probably salts; 31, unknown; A, B, C, and D, unknowns.

ninhydrin-unreactive, S^{35}-containing compound appeared in very small amount at position A of both figures 3 and 4. Three other S^{35}-containing compounds appeared in figure 4 but not in figure 3. These were B, C, and D. These spots also were very weak and represented only a small percentage of the total radioactivity. They were found in these fractions because these autoradiographs were exposed over a long period of time. All these substances (A, B, C, and D) may be organic sulphur compounds in view of their mobility. There is clearly a great variety of substances that could be considered as possibilities (5).

Some consideration was given to the possibility that one or other of the unknown spots of figures 3 or 4 might be due to taurine (a decarboxylation product of cysteic acid). However, none of these satisfy all the require-

TABLE I

THE S^{35} ACTIVITY OF THE AREAS ATTRIBUTABLE TO CYSTINE AND METHIONINE DERIVATIVES AND GLUTATHIONE ON PAPER CHROMATOGRAMS OF ALFALFA LEAF FRACTIONS. DATA SHOW ACTIVITY MEASURED AS A PERCENTAGE OF TOTAL PLACED ON THE CHROMATOGRAM.

	Cysteic acid	Activity in region of cystine	Total cystine	Glutathione	Methionine sulphoxide	Methionine sulphone	Total methionine	Total* activity recovered
Water extract free of protein	70	...	70	7	...	...	...	77
Hydrolysate of alcohol-insoluble fraction**	15	2	17	...	9	42	51	68
	9	...	9	...	15	35	50	59
	7	4	11	...	50	...	50	61
Hydrolysate of heat coagulum	30	12	42	...	12	...	12	54
Hydrolysate of water-alcohol- and acid-insoluble fraction**	2	10	12	...	52	...	52	64
	...	12	12	...	30	...	30	42

* Total cystine + total methionine + glutathione.

** Replicate chromatograms.

ments either as to position, to radioactivity, or to ninhydrin reactivity. It may be stated here that all attempts to find taurine in these alfalfa preparations have failed and it should, therefore, be concluded that it does not normally occur either as a metabolite or as a decomposition product of the extracts.

The extent to which the sulphur-containing compounds may be accounted for in terms of cystine and methionine derivatives and glutathione, is clearly shown in table I. It should be noted that owing to absorption of the weak S^{35} radiation by the paper, the recovery values in table I are necessarily low. If equal amounts of activity are spread over unequal areas of paper, the more concentrated spot will show a larger count than the others. From such experiments it is estimated that the values in table I may be as much as 20 to 30% low.

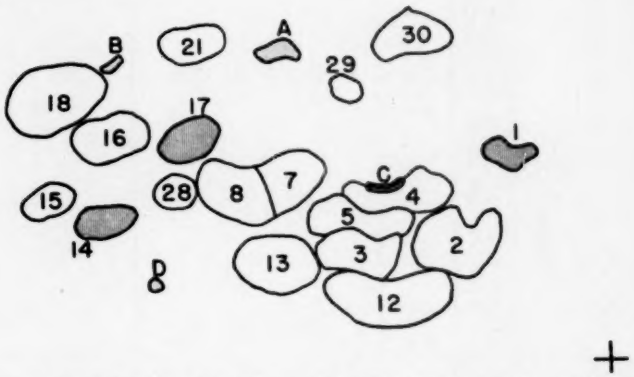


FIG. 4. Key to amino and S^{35} compounds in the hydrolysate of the water-, alcohol-, and dilute-acid-insoluble fraction of alfalfa leaf. Legend same as for figure 3.

Discussion

The amino acids found both "free" and in proteins in alfalfa leaves are similar to those found in other tissues (**6**). No other comparable analyses of amino acids of alfalfa leaves have been found. Legume roots have been found by VIRTANEN to excrete aspartic acid, β-alanine (**11**) and glutamic acid (**12**) so there are presumably large quantities of these in the roots. It is of interest, therefore, that β-alanine is found in the leaf extracts; indicating that it is not peculiar to the root nodules. ZELITCH, BURRIS, and WILSON (**14**) and HUNT (**3**) have each reported a range of amino acids in legume roots similar to that described in this study.

In regard to the sulphur compounds, it is surprising that there were only two alcohol-soluble sulphur compounds—cystine (as cysteic acid) and glutathione, and that there was no indication of methionine or any of its oxidation products. In the hydrolysates, however, methionine (as its oxidation

products) was present. Even when the samples were not treated with hydrogen peroxide, it was impossible to determine whether the methionine occurred in the plant only as methionine since it may have been oxidized in the course of chromatography (**1**). It is of interest to note from table I that methionine predominated over cystine in the insoluble proteins. The reverse was true in the heat coagulum.

Clearly, there are not many organic sulphur compounds in leaves other than the sulphur amino acids. This is borne out by the chromatograms which were scanned with a Geiger counter and the areas of activity compared with the ninhydrin-reactive regions (see table I). As much as 70 to 75% of the activity placed on the paper could be accounted for in terms of ninhydrin-reacting substances (Nos. 1, 14, 17, and 34 of figs. 1 B, 2 B, 3 and 4). Considering the number of possible sulphur compounds which might occur, it is somewhat surprising that so much of the S^{35}, as shown by both the scanned chromatograms and autoradiographs, was present in so few compounds. However, some of the radioactivity due to immobile substances and previously attributed to inorganic forms of sulphur may have been due to sulphonic acids or other polar organic molecules.

It was of interest to note again (**6**) that γ-amino butyric acid did not occur in the protein but was found only in the free state.

Summary

1. The free and combined amino acids of alfalfa leaves have been examined by the methods of paper chromatography and autoradiography for S^{35} which was introduced into the plants in the form of sulphate via the roots.

2. Aspartic and glutamic acids, alanine and serine were the conspicuous amino acids which were detected free in alfalfa, and the amides glutamine and asparagine were also prominent.

Valine, the leucines, and tyrosine were all readily detectable though they occurred in smaller amounts, relative to the others, than in many other tissues (*e.g.*, potato tuber). The only free basic amino acid detected was arginine.

γ-Amino butyric acid occurred, though it was not as conspicuous in alfalfa as in many other plants.

The free sulphur-containing compounds consisted of cystine (detected as cysteic acid) and substances, most probably glutathione, which yielded cystine on hydrolysis.

Methionine and its oxidation products, the sulphoxide and sulphone, were not detectable in the free state.

3. The combined amino acids of alfalfa were examined after hydrolysis of (a) an alcohol-insoluble fraction, (b) a heat coagulum of a water extract, and (c) a water-, alcohol-, and acid-insoluble fraction. These amino acids were those commonly found in proteins; valine and the leucines were more prominent in the hydrolysates than in the free state; glycine, lysine, threonine, methionine, and proline were only detected in the protein hydrolysates.

Hydroxyproline appeared only in the hydrolysate of the water-, alcohol-, and acid-insoluble fraction. Phenylalanine was detected only in the hydrolysate of the heat-coagulable protein.

Certain unidentified, sulphur-free, but ninhydrin-reactive substances occurred in the acid hydrolytic products of alcohol-insoluble material.

The prominent and identifiable S^{35}-containing compounds in these hydrolysates were methionine sulphone and sulphoxide from methionine and cysteic acid from cystine. Certain sulphur compounds, present in small amount in the hydrolytic products, were detected by autoradiographs of chromatograms. None of these satisfied the requirements for taurine.

For the work at the Department of Botany, University of Rochester, we wish to acknowledge facilities furnished out of a grant to one of us (F. C. S.) from the Nutrition Foundation. During the course of the work F. K. Millar was an A.E.C. Predoctoral Fellow in the Biological Sciences of the National Research Council.

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HORMONE-INDUCED PARTHENOCARPY IN THE CALIMYRNA FIG AND A COMPARISON OF PARTHENOCARPIC AND CAPRIFIED SYCONIA

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(WITH THREE FIGURES)

Received May 15, 1950

Introduction

The problems associated with caprification (pollination) of Calimyrna figs and the desirability of standardizing or, still better, eliminating this cultural operation have been discussed (**5**). Because of its relatively low cost, low required concentration, and complete lack of injurious effects on fruit and foliage, para-chlorophenoxyacetic acid was found, during the season of 1948, to offer considerable promise as a material to induce parthenocarpy and, thereby, substitute for caprification of the Calimyrna fig (**4**). Commercial exploratory trials during the season of 1949 demonstrated the practicability of using this material as a substitute for cross-pollination by the fig wasp. The extent to which the industry adopts this practice depends mainly upon the demand by the consuming public for a fig that does not contain sclerified achenes ("seeds") and lacks the "nutty" flavor which characterizes caprified Calimyrna figs.

Although the largest percentage of figs set in the 1948 investigation was obtained with an aqueous spray containing 60 p.p.m. of para-chlorophenoxyacetic acid (**4**), further information was desired on the range in concentration of this compound effective in promoting parthenocarpy and the thoroughness with which the spray should be applied. Tests with Sure Set, a proprietary material containing para-chlorophenoxyacetic acid, para-fluorophenoxyacetic acid, and 2,4,5-trichlorophenoxyacetic acid were also made and will be discussed later.

The commercial trials mentioned above enabled the periodic collection of samples large enough to make certain physical and chemical comparisons between induced parthenocarpic and caprified figs.

Materials and methods

Parthenocarpic fig samples were obtained from a 20-acre orchard that was sprayed on June 21, 1949, with a water solution containing 60 p.p.m. of the ammonium salt of para-chlorophenoxyacetic acid (PCPA). The solution was applied with a Robinson Fan Sprayer at the rate of about 275 gallons per acre. Samples consisting of 100 figs, picked at random from the proximalmost nodes on as many shoots, were collected three times during the fruit-growth period. An equal number was also collected from an adjacent caprified orchard. The first samples were collected on June 21, the

day on which the spray was applied; on July 20; and on August 16, when the figs were mature. In addition to the notation of differences in color, the following measurements were made: volume, fresh weight, transverse and polar diameters, thickness of receptacular tissue, and diameter of floral cavity. Sugar determinations were made on both types of figs at maturity only.

To determine the range of concentration at which PCPA is effective in inducing parthenocarpy, as well as to carry on other incidental studies, an isolated Calimyrna orchard was chosen for test purposes. Because this particular orchard was four miles distant from the nearest caprified Calimyrna orchard, covering the test branches as was done previously (**4, 5**) with cloth bags to exclude the pollen-carrying fig wasp was unnecessary. The materials tested were applied on June 21, 1949, with a continuous-delivery type, hand-operated fly-spray gun. Both the leaves and the young syconia were sprayed unless otherwise specified. Ten branches were used per treatment, each bearing at least five syconia when the growth regulators were applied. As a basis for comparing the percentage of syconia set as a result of growth-regulator application, determinations of set were made in three orchards that had been caprified in the regular commercial manner.

In a previous paper (**3**), it was reported that 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) sprays at concentrations of 10 p.p.m. or greater not only induced parthenocarpy but greatly hastened fruit maturity, although they severely damaged or even killed the sprayed branches. Because accelerating the maturity of developing fruits is of both fundamental and practical significance, further studies were made with 2,4,5-T in an attempt to eliminate the undesirable reactions. This compound was used alone at various concentrations and in combination with PCPA.

Results and discussion

An examination of the data relating the concentration of PCPA to percentage of fruit set (fig. 1 and table I) clearly shows a progressive increase in set to a maximum at 80 p.p.m. of PCPA, beyond which the values decrease. However, even at a concentration of 100 p.p.m., over 10% more syconia set parthenocarpically than the average for the three caprified orchards. These results indicate that from a practical standpoint there is a fairly wide range of PCPA concentration that will produce a satisfactory set. The relationship shown in figure 1 apparently is typical for most hormone-induced responses (**7**), wherein there is an increase in response with increasing concentration to a maximum beyond which inhibition or toxicity occurs.

A spray containing 60 p.p.m. of PCPA applied to actively growing fig trees has been observed to cause considerable epinasty and chlorosis of the leaves and curling of the distal portions of the shoots. Fortunately, to induce parthenocarpy in the Calimyrna fig, the time at which the growth regulator sprays are applied is somewhat after the growth of the shoots has

ceased and the leaves have fully expanded. As a consequence, there were no observable symptoms of leaf, fruit, or shoot injury accompanying any of the concentrations of PCPA used. The rather wide range in concentration of PCPA effective in promoting parthenocarpy in the Calimyrna fig, together with the fact that no injury accompanies its use even at a concentration of 200 p.p.m., emphasizes the possibility of using this material commercially.

The comparative development of caprified and induced parthenocarpic syconia, as determined by certain physical measurements, is typified by the data in table II. Samples were collected and measured on June 21, the day the spray was applied, to determine the differences, if any, between syconia

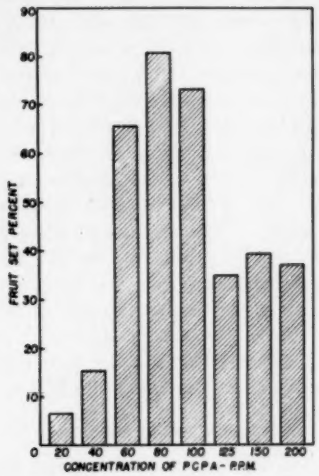

FIG. 1. Effect of concentration of para-chlorophenoxyacetic acid applied during the caprification period upon fruit set in the Calimyrna fig.

that were destined to develop parthenocarpically and those that were to develop as a result of caprification. The data in table II bear out the fact that syconia from the two orchards on this date were practically identical in all respects. However, by July 20, about a month before maturity, both external and internal differences were evident in the two types. Externally, the parthenocarpic syconia were considerably more ribbed than those caprified (fig. 2). The ribs were so prominent on some that the surface was corrugated. Although this condition persisted to a less degree at maturity, it was a distinguishing characteristic of the parthenocarpic fruits even after they were dried. In addition, the necks of these fruits were somewhat flattened. Upon maturity the parthenocarpic fruits were light lemon yellow in color while those caprified were typically golden yellow.

Internally, particularly upon maturity, the two types of figs were markedly different, the most pronounced differences being in the color of the pulp and in the development of the achenes (figs. 2 and 3). The pulp in caprified figs at maturity was of a light strawberry red color while that in the parthenocarpic fruits was a light amber yellow. Although the red pigmentation is confined to the outer ovary wall of the individual achenes, the latter are dispersed so thickly throughout the pulp in a thoroughly caprified

TABLE I

THE EFFECT OF CERTAIN GROWTH REGULATORS APPLIED AT VARIOUS CONCENTRATIONS UPON FRUIT SET IN THE CALIMYRNA FIG

Material or treatment	Concentration	Fruit set
	p.p.m.	%
Para-chlorophenoxyacetic acid	20	6.5
	40	15.4
	60	65.8
	80	81.0
	100	73.2
	125	35.0
	150	39.5
	200	37.2
Sure Set (Dow Chemical Co.)*	25	34.9
	50	31.0
	75	54.8
	100	57.8
Para-fluorophenoxyacetic acid	25	0.0
	50	33.3
	100	88.0
Para-chlorophenoxyacetic acid		
Applied to leaves only	60	71.7
Applied to fruits only	60	31.1
Caprified Controls		
Orchard 1		58.5
Orchard 2		65.8
Orchard 3		67.4
Average		63.9

*Sure Set contains para-chlorophenoxyacetic acid in a water-miscible solvent.

fig that the entire area appears light strawberry red in color. On the other hand, in syconia in which only a portion of the flowers are pollinated, the red pigmentation is confined to that area of the pulp containing the fertilized achenes (fig. 3).

Sclerification of the ovary walls of achenes had taken place by July 20 in caprified figs, whereas none had occurred in parthenocarpic fruits even at maturity, the latter being the so-called "seedless" figs. Apparently, the formation of red pigments is associated with the development of the ovary wall of the achene, since the pulp of parthenocarpic figs contains no red

color and since this coloration in the pulp of caprified figs is limited to areas in the syconium where the flowers are pollinated.

Caprified and parthenocarpic fruits were practically identical in average volume and diameter measurements (table II). Parthenocarpic figs at maturity, however, weighed about three grams (7%) less and were somewhat more oblate in form than caprified figs. Of particular interest is the differential development of the receptacular tissue in the two types of fruit. At the time the young syconia were either pollinated or sprayed with PCPA, the thickness of the receptacular tissue was approximately the same in both types. From that time on, this tissue in caprified syconia progressively decreased in thickness from 2.83 mm. to 2.11 mm. The receptacular tissue in parthenocarpic syconia, on the other hand, progressively increased in thickness from 2.74 mm. to 4.58 mm. at maturity. This tissue was more than

TABLE II

COMPARATIVE MEASUREMENTS OF CAPRIFIED AND PARTHENOCARPIC CALIMYRNA FIG SYCONIA PRODUCED WITH A SPRAY APPLICATION OF 60 P.P.M. OF PARA-CHLOROPHENOXYACETIC ACID

Type of syconia and date of sampling	Volume	Fresh weight	Transverse diameter	Polar diameter	Thickness of receptacle	Diameter of floral cavity
	cc.	*gm.*	*mm.*	*mm.*	*mm.*	*mm.*
June 21						
Caprified	11.71	6.76	25.36	21.61	2.83	19.70
Parthenocarpic	11.95	6.84	25.42	21.45	2.74	19.94
July 20						
Caprified	14.94	12.28	32.42	25.90	2.52	27.38
Parthenocarpic	15.13	10.59	33.07	24.03	3.71	25.66
August 16*						
Caprified	46.00	45.68	48.64	34.62	2.11	44.42
Parthenocarpic	46.30	42.48	49.48	33.10	4.58	40.30

*Syconia mature on this date.

twice as thick in parthenocarpic as in caprified figs, thus accounting for the smaller diameter of floral cavity in the former. A histological examination of caprified and parthenocarpic Calimyrna fig syconia made by BASKAYA and CRANE (1) revealed that the receptacular tissue in the latter contained somewhat larger cells and more cell layers than the former.

The receptacular tissue of the fig syconium is composed of parenchyma cells that function primarily for storage of elaborated food materials. Apparently, in caprified syconia these materials are gradually translocated from the parenchyma tissue of the receptacle into the developing achenes. Since the development of achenes in parthenocarpic syconia is incomplete (1), elaborated food materials that otherwise would be utilized by the achenes may accumulate in the parenchyma of the receptacle. This process may cause the receptacular tissue to thicken progressively with development of the syconia to maturity.

Parthenocarpic figs contained 4.5% more total sugars than did caprified fruits, substantiating previous investigations on the fig (**4**) and the tomato (**6**). On a dry weight basis, mature caprified and PCPA-induced parthenocarpic figs contained 74.5 and 79.0% total sugars, respectively.

The differential development of the receptacular tissue in caprified and parthenocarpic figs offers a partial explanation for the fact that the latter contain a higher percentage of sugar than the former. As mentioned above, the parenchyma in the receptacle is primarily storage tissue; since the ratio of this tissue to the remainder of the syconium is larger in parthenocarpic

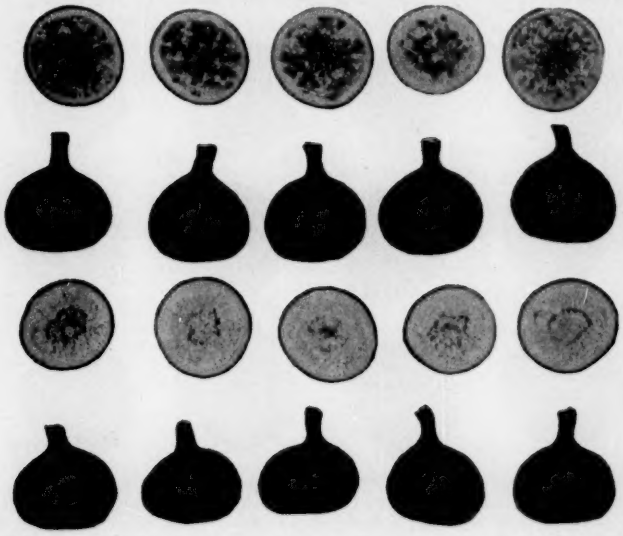

FIG. 2. *Top,* Calimyrna figs produced by the normal process of caprification; *bottom,* parthenocarpic Calimyrna figs produced with 60 p.p.m. of the ammonium salt of para-chlorophenoxyacetic acid. Samples collected on July 20, 1949, one month after caprification and spraying.

than in caprified syconia, the difference in sugar content may be explained on this basis.

A spray of PCPA applied only to the leaves induced more than twice as many syconia to set parthenocarpically as when it was applied to the young syconia only (table I). The "transmission of effect" of naphthaleneacetic acid on apple drop, as reported by BATJER and THOMPSON (**2**), is demonstrated clearly here in the use of PCPA to induce parthenocarpy in the fig. This response is in direct contrast to that obtained in 1948 with gamma-(indole-3)-*n*-butyric acid (**4**).

The difference in response when PCPA and IBA were applied to either the leaves or young syconia is of interest. Factors such as differential rate

and degree of absorption of the growth regulators by the leaves and syconia, translocation of the materials within the plant to regions where utilized, and inherent differences in the potency of the chemicals themselves are undoubtedly of much importance in this respect.

The application of Sure Set at various concentrations gave a trend in parthenocarpic fruit set similar to that obtained with PCPA (table I). Sure Set, however, appears to be somewhat less effective in promoting parthenocarpy in the Calimyrna fig than PCPA when used at identical concentrations. This discrepancy is not understood but may be the result of a difference in degree of absorption of the two materials by the leaves.

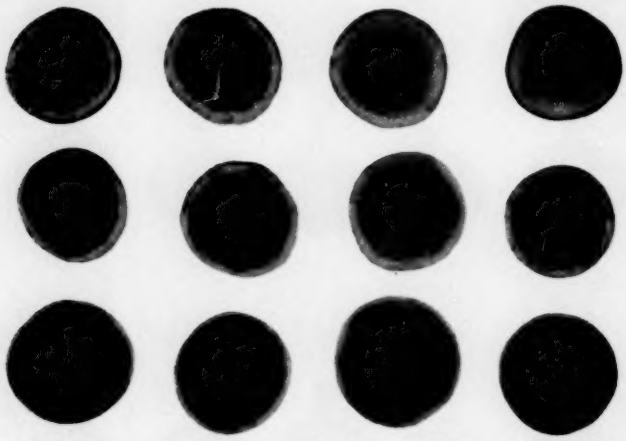

FIG. 3. Cross sections of parthenocarpic and caprified Calimyrna fig syconia. *Top*, parthenocarpic induced by 60 p.p.m. of ammonium salt of para-chlorophenoxyacetic acid; *middle*, partially caprified; *bottom*, fully caprified. Note the difference in thickness of the receptacular tissue in the three samples of syconia, as well as differences in red pigmentation indicated by the darkness of the pulp. Samples collected on August 16, 1949, at maturity.

Para-fluorophenoxyacetic acid appears to be equally as effective in promoting parthenocarpy as PCPA, an 88% fruit set being obtained with a concentration of 100 p.p.m. of this material (table I). Here, likewise, there were no deleterious effects of foliage, fruits, or shoots from the use of this material.

Concentrations of 2,4,5-T less than 10 p.p.m. did not induce parthenocarpy, but at or above this concentration, parthenocarpic fruit maturation occurred about two weeks after application. When 2,4,5-T was combined with PCPA at 60 p.p.m., the syconia were invariably set, but acceleration of maturation did not occur unless the concentration of 2,4,5-T was 10 p.p.m. or greater. Hastening of maturity, however, was always accompanied by

leaf chlorosis and injury to the shoots in varying degree, depending upon the concentration of 2,4,5-T, and was not significantly modified by the presence of the other hormone.

The toxic effect of 2,4,5-T apparently is not separable from the property of inducing parthenocarpy by any means so far tried, at least with the Calimyrna fig. The difference in concentration between a spray that will set fruit and one that will result in injury is small or non-existent. This is not an uncommon phenomenon; *e.g.*, the well known deleterious effects often occurring when 2,4-D or PCPA is sprayed on tomato plants in sufficient quantities to induce fruit set.

The data presented in table III serve to emphasize how relatively small differences in molecular structure may modify physiological response. Three closely related compounds—2,4-D, 2,4,5-T and PCPA—all will set syconia parthenocarpically in the Calimyrna fig, all will cause an acceleration in maturation, and all will cause injury. Yet the concentration required for each chemical to bring about each of these reactions is very different.

TABLE III

THE RELATIONSHIP BETWEEN CONCENTRATION OF CHLORINATED PHENOXY ACIDS AND INDUCED RESPONSE

Acid	Minimum concentration in p.p.m. required for:		
	Inducing parthenocarpy	Accelerated maturation	Injury
2,4-D*	250	250	250
2,4,5-T	10	10	10
PCPA	20–40	250	250

*See literature cited (8).

Although the reason for these differences is not known, certain conclusions are evident. With 2,4-D or 2,4,5-T, there is no difference in concentration between that which will cause the syconia to set and that which will bring about injury, whereas with PCPA this difference is of considerable magnitude. It is also apparent that when acceleration of maturity occurs, injury also results.

Summary

With a progressive increase in concentration of para-chlorophenoxyacetic acid to 80 p.p.m., there was a progressive increase in the percentage of syconia induced to set parthenocarpically, 17% more being set at the 80 p.p.m. concentration than on the caprified controls. Concentrations above 80 p.p.m. resulted in a depressing effect upon the percentage of syconia that were set. The rather wide range in concentration of para-chlorophenoxyacetic acid found to be effective in promoting parthenocarpy without injury to syconia, leaves, or shoots emphasizes the commercial possibility of this material for use by the fig industry.

A comparison of caprified and parthenocarpic Calimyrna figs produced with 60 p.p.m. of para-chlorophenoxyacetic acid revealed that the two types were not markedly different with respect to volume and diameter. Upon maturity, parthenocarpic fruits were somewhat more ribbed and lighter in color than caprified fruits. Parthenocarpic fruits weighed about 7% less than caprified fruits, and were somewhat more oblate in form. The pulp in caprified figs was of a light strawberry red color, while that in parthenocarpic fruits was a light amber yellow. In contrast to caprified figs, where sclerification of the inner ovary wall or endocarp of the achenes took place a month or more before maturity, no sclerification of this tissue was evident at maturity in parthenocarpic figs. The width of the receptacular tissue progressively decreased with development of caprified syconia to maturity, whereas in parthenocarpic syconia this tissue progressively increased in thickness, when at maturity it was twice as thick as in caprified fruits.

As a consequence of rate and degree of absorption or translocation within the plant, para-chlorophenoxyacetic acid was much more effective in promoting parthenocarpy when applied to the leaves than when applied to the young syconia.

Para-fluorophenoxyacetic acid was found to be as effective in promoting parthenocarpy as para-chlorophenoxyacetic acid. The application of this material likewise resulted in no deleterious effects on foliage, syconia or shoots.

With 2,4,5-T alone or in combination with PCPA, it was not possible to obtain accelerated fruit maturation without accompanying toxicity to the foliage.

Acknowledgment is gratefully made to the Shell Oil Company and the Dow Chemical Company for supplying the growth regulators used in this investigation.

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EFFECTS OF SOIL TEMPERATURE AND LIGHT INTENSITY ON ROOT GROWTH OF LOBLOLLY PINE SEEDLINGS

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(WITH NINE FIGURES)

Received May 22, 1950

The experiments described in this paper were planned to test the effects of certain environmental factors on the root growth of loblolly pine seedlings. TURNER (**26**) and REED (**22**) studied the seasonal periodicity of root growth of this species under field conditions, but no measurements were made under controlled conditions. Reed noted a marked reduction in root growth during the time when soil temperatures were lowest and also at times when the soil was driest. Turner's data also indicate this same general trend. It was, therefore, decided to measure root growth of loblolly pine under controlled conditions to find whether or not a definite and perhaps quantitative relationship to soil temperature and other factors could be shown.

Materials and methods

Two series of experiments were performed, the first during the spring and summer of 1941, the second during the winter of 1946. The seedlings used in 1941 were grown from seed collected in North Carolina in 1940, and the seedlings used in 1946 were grown from seed collected in Louisiana in 1945.

The seedlings were grown in glass tubes about two inches in diameter and 18 inches long. These tubes were closed at the lower end with rubber stoppers and a layer of cinders placed over the stoppers. The tubes were then filled with a loamy greenhouse soil, which was kept wetted to field capacity. Although the soil was heated in an electric sterilizer before use, it still contained two species of small animals, a nematode and a collembolan. Neither seemed to be harmful; apparently they fed only on dead tissue sloughing off the roots. The collembolans may have aided in aeration by opening small passageways in the soil.

The soil atmosphere in the tubes was analyzed with a Haldane apparatus and found to have approximately the same concentration of oxygen as the outside air. The concentration of carbon dioxide in the soil was somewhat higher than outside. The average amount was 1.0% and the maximum 2.88%. CANNON and FREE (**6**) reported that for several species there was no detrimental effect on root growth of 20% or more carbon dioxide if oxygen were not deficient. Since the soil atmosphere normally contains more carbon dioxide than ordinary atmosphere and excesses from 0.2 to 5% are not unusual (**28**), it is believed that growth was not abnormally influenced by deficient oxygen or by an excess of carbon dioxide.

In the first series of experiments, the seedlings were started from seeds planted in the tubes, but this proved unsatisfactory because the seeds did not germinate uniformly. Seeds for the second series of experiments were germinated on moist sand, and three seedlings were transplanted to each tube. About two weeks at 20° C were allowed for the seedlings to become established before an experiment was started. The tubes were supported in racks at an angle of about 40 degrees. This caused the roots to grow along the lower side of the tubes where they were visible and could be measured. Measurements were made for a period of three weeks for each series of tests.

Soil temperature was controlled by immersing the racks and tubes in large tanks of water equipped with refrigeration and heating units, controlled by thermoregulators and relays. To insure uniform temperature throughout, each tank was equipped with a stirring unit which operated intermittently. The variation in temperature was less than one degree centigrade.

Four tanks were operated simultaneously. The tanks were covered with pieces of insulating board containing holes for 12 tubes. Even though these covers were not entirely light-tight, not enough light entered to permit the growth of green algae in the tubes, and it is, therefore, believed that such a low intensity could have had no significant effect on root growth. Although considerable variations in air temperature and humidity occurred in the greenhouse room where the tanks were located, all plants in a series were exposed to the same variations.

Effect of soil temperature on root growth

Selection of temperatures

The range of temperatures selected was by 5-degree intervals from 5° C to 35° C. Four series of tests were made. Two of the series were for soil temperatures of 5° C, 10° C, 15° C and 20° C and two were for soil temperatures of 20° C, 25° C, 30° C, and 35° C. Zero centigrade was omitted because it was assumed that growth would be extremely slow at this temperature and that it would be difficult to prevent the soil water from freezing and bursting the tubes. Temperatures in excess of 35° C were not tested since this temperature is close to the reported lethal high temperature of 44.3° C for the roots of other conifers (after 5-hour exposures) (**25**).

Root measurements

At least three days were allowed after the water had reached the desired temperature before measurements of growth were made. The daily measurements were made by artificial light at night to avoid exposing the roots to sunlight. A short line was scribed with a carborundum pencil on the glass tube near the root tip. Growth was recorded by measuring the distance from the root tip to this index mark. Measurements were made with a binocular microscope attached to a base having a movable stage similar to that described by Patterson (**20**). Movement of the stage was

recorded to the nearest one-hundredth millimeter through direct coupling with a dial micrometer.

Results

Since between 200 and 500 individual measurements of root growth were made for each temperature tested the results presented here must be limited to the averages calculated for each test. The individual measurements are on file in the library of Duke University. Root growth at 35° C during the first five days of measurement is not included in this analysis because growth did not reach a stable rate during this period and therefore could not be considered a linear function of time. The data for the first five days at 35° C are considered in the section on temperature changes.

Series one and two supplied data for the estimates of average daily growth for each temperature from 5° C to 35° C for seedlings grown from seed collected in North Carolina. There was a small difference in the rate at 20° C between the two series. This was probably due partly to season and was not statistically significant. Thus, the effect of temperature on root growth may be considered as a continuous series from 5° C to 35° C (fig. 1). As the temperature increased there was a fairly uniform increase

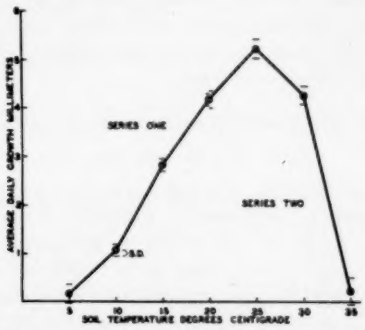

Fig. 1. Relationship between average daily root growth (b) in millimeters and temperature in degrees centigrade for loblolly pine seedlings. North Carolina seed source.

in growth rate from 0.17 ± 0.19 mm. per day at 5° C until it reached a maximum of 5.23 ± 0.19 mm. per day at 25° C and then it rapidly decreased. The rate at 35° C of 0.23 ± 0.29 mm. per day was only slightly greater than the rate at 5° C.

Series three and four also supplied data for an estimate of average daily growth rate for each temperature from 5° C to 35° C. However, the seedlings tested in these series differed from those of series one and two not only because they were grown during a different season of the year, but also because seeds were collected in Louisiana. Although the seasonal difference between the two series appeared to be somewhat greater as indicated by the

two estimates of the average daily root growth for 20° C, again it was not statistically significant. Thus, these two series may also be considered as continuous (fig. 2).

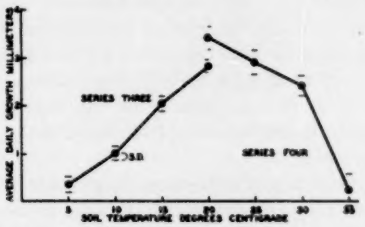

FIG. 2. Relationship between average daily root growth (b) in millimeters and temperature in degrees centigrade for loblolly pine seedlings. Louisiana seed source.

As the temperature increased, the daily rate of root growth in these two series also increased rather uniformly from 0.36 ± 0.16 mm. per day at 5° C to a maximum of 3.41 ± 0.24 mm. per day at 20° C and then decreased to 0.24 ± 0.34 mm. per day at 35° C. The maximum growth rate not only occurred at a lower temperature than was found in series two, but also was smaller. The decrease in growth rate as the temperature was increased above the optimum was not so abrupt.

The calculated values of the average daily root growth (b) for the four series are given in table I.

TABLE I

AVERAGE DAILY ROOT GROWTH AND STANDARD ERROR IN MILLIMETERS FOR EACH TEMPERATURE TESTED

Temperature	Series 1	Series 2	Series 3	Series 4
5° C	0.17 ± 0.19		0.36 ± 0.16	
10° C	1.07 ± 0.12		1.01 ± 0.15	
15° C	2.83 ± 0.13		2.04 ± 0.15	
20° C	4.18 ± 0.12	4.18 ± 0.17	2.83 ± 0.13	3.41 ± 0.24
25° C		5.23 ± 0.19		2.90 ± 0.25
30° C		4.27 ± 0.18		2.41 ± 0.21
35° C		0.23 ± 0.29		0.24 ± 0.34

The calculation of the average daily root growth was based on the assumption that the length of any individual root can be expressed as a linear function of time; or that there is a uniform increase in length with each unit increase in time. If this is valid, then the coefficient, b, of the regression equation

$$Y = \overline{y} + b\,(x - \overline{x})$$

derived from the observed values of length (y) and time (x) is the average response of root growth to a given temperature based on all the observations

at that temperature. (The symbols $\bar{y}$ and $\bar{x}$ represent the mean of all observed y and x values respectively.)

The validity of the assumption and the significance of the differences in the calculated values of b for each temperature may be tested through the method of least squares in the analysis of variance and covariance.

Because the experiments were conducted in four series, each consisting of 20° C and three higher or three lower temperatures and differing in time and source of seed, it seems appropriate that the tests of differences should be between the several temperatures of each series rather than grouping all the data for all the series.

The tests of significance of differences in average daily root growth in response to different temperatures yield variance ratios which exceed the expected values at the 0.1% level of significance. Thus, the observed differences between average daily root growth for the temperatures tested could be expected to occur by chance less than one time in 1,000.

Effect of soil temperature change on root growth

The rate of daily root growth at 35° C showed a very marked time effect. It was high when the first measurements were made but the rate decreased rapidly to a low fairly constant rate after about five days at 35° C. This response to high temperature can be expressed by the exponential regression equations:

$$Y = 8.09 \div (1.143)^{\frac{1}{x}}$$

and

$$Y = 2.22 \div (1.156)^{\frac{1}{x}}$$

for the two experiments at 35° C. Y is the average daily growth in millimeters and x the time in days from start of measurements at the specified temperature.

These equations do not differ appreciably in slope. The difference in level of the Y intercept probably can be attributed to a difference in time between the change in temperature and the first measurements of root growth at the new temperature.

A similar diminution has been reported by LEHENBAUER (**17**) in the growth rate of maize shoots exposed to temperatures above 31° C for prolonged periods. LEITCH (**18**) observed that the growth of pea roots followed this same pattern above 40° C, but between 30° C and 35° C the rate decreased during the first half hour, then a secondary, temporary increase in rate appeared. This secondary increase in rate reached a maximum ½ to 1½ hours after the temperature was changed and then the rate decreased uniformly and rather slowly.

ASKENASY (**2**) studied the growth rate of maize roots when subjected to gradual or sudden cooling from moderate temperatures. He found that the rate decreased concurrently with decreased temperature, but resumption of

growth rate with increased temperatures was dependent on how nearly the low temperature used had approached the minimum temperature for growth of the species. The more nearly the low temperature approached the minimum for growth the slower the rate of recovery when the temperature was again raised. He also noted that sudden cooling caused an apparent contraction of roots.

Methods and results

Two experiments were performed to study the effect of temperature changes on the growth rate of loblolly pine roots. In the first experiment the temperature was changed by transferring 10 seedlings from the 20° C waterbath to the one at 35° C and measuring the increase in length at 15-minute intervals for the first two hours. Subsequent measurements were made three, five, and 29½ hours after the transfer.

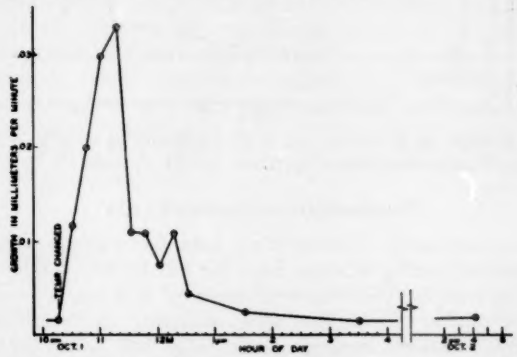

Fig. 3. Average rate of root elongation of loblolly pine seedlings in millimeters per minute following a sudden change in temperature from 20° C to 35° C.

It was estimated by measurements with a thermocouple adjacent to the inner wall of the tube that 15 minutes were required for the roots to reach the new temperature after transfer.

Following the temperature change the rate of elongation per minute increased regularly to a maximum of 0.0328 mm. at the end of 61 minutes and then decreased to 0.0075 mm. after 106 minutes. There was a secondary and temporary increase to 0.0108 mm. per minute at the end of 121 minutes after which elongation decreased slowly to 0.0019 mm. per minute 30 hours after transfer. The final rate per minute is equivalent to a daily rate of 2.736 mm. (fig. 3). The characteristics of this curve are similar to that described by Leitch (**18**).

For the second experiment 15 seedlings which had been growing one week in soil at 35° C were transferred to the 20° C waterbath. At the time of

transfer the average growth rate, calculated to the nearest one-hundredth millimeter, was 0.00 mm. per hour. One hour after transfer a very slight average increase in length occurred followed by a slight decrease during the next two hours. These minor changes might possibly have been the result of slight turgor changes associated with the shock of the temperature change. Very little growth occurred during the next two days. At the end of the second day after transfer one root had grown 0.21 mm. and ten hours later a second root had grown 0.27 mm. Six and one-quarter days after the transfer all roots were growing at an average rate of 0.037 mm. per hour. During the next hour the rate increased to 0.100 mm. per hour, but 17 hours later the rate had decreased to 0.050 mm. per hour (fig. 4).

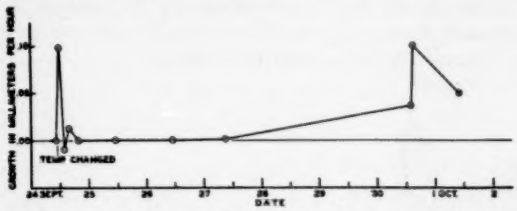

FIG. 4. Average rate of root elongation of loblolly pine seedlings in millimeters per hour following a sudden change in temperature from 35° C to 20° C.

Temperature and leaf–root ratio

Following each series of temperature tests, the seedlings were removed from the soil by soaking in water until the soil fell away from the roots. The seedlings from the first tests were preserved in a weak formalin–acetic acid–alcohol solution until all tests were completed. At the end of the temperature experiments the seedlings were dried at 102° C and the weights of the roots, stems and leaves were determined on an analytical balance.

All chlorophyll-bearing organs have been designated "leaves." These include cotyledons, new shoot growth, and leaves, but do not include the hypocotyl. Leaf weight, thus, represents all the chlorophyll-bearing tissue and, except for the cotyledons, it is almost entirely shoot growth which was made during the tests. Although there was a very small amount of stem growth during the tests, most of the weight of the stem was the result of growth prior to measurements of root growth, and for this reason was not included.

RESULTS

Average leaf–root ratios for the seedlings grown from North Carolina seed decreased with increased temperature from 3.92 ± 0.03 at 5° C to a minimum of 2.08 ± 0.02 at 20° C. Above 20° C there was an increase with higher temperatures. The leaf–root ratio at 30° C was 4.19 ± 0.12, and there was a rather abrupt increase to 8.36 ± 0.25 at 35° C.

Leaf–root ratios of seedlings produced from Louisiana seed decreased

with increased temperature from 4.81 ± 0.10 at 5° C to a minimum of 2.85 ± 0.02 at 20° C and then increased with higher temperature to 4.57 ± 0.02 at 30° C. In contrast to the North Carolina seedlings, the leaf–root ratio of 4.39 ± 0.03 at 35° C was slightly less than at 30° C. Since there was little root growth at 35° C the differences between the leaf–root ratios for the two seed sources may indicate a more branched root system was produced by the Louisiana seedlings during the establishment period at 20° C.

The effect of temperature on leaf–root ratio of loblolly pine is similar to the top–root ratios reported by ADAMS (1) for 1-year-old white pine seedlings. He found the lowest top–root ratio when the mean soil temperature ranged from 55° F at an 8-inch depth to 88° F at the surface.

Daily periodicity

Daily periodicity in growth rate of roots and shoots of various plants has long been known. In general, studies of periodicity have shown that maximum growth occurs during evening or night and minimum growth during early morning or forenoon (9, 23).

To determine whether the same situation exists in loblolly pine roots, measurements of the growth of 21 roots were made at 2-hour intervals for a 24-hour period on June 14 and 15, 1941. The roots were growing in a soil temperature of 25° C. A record of relative humidity for this period was obtained by means of a hair hygrograph.

RESULTS

The average root growth of the 21 roots plotted on average time (hour and minute) of measurement for each 2-hour interval and the relative humidity at the end of each 2-hour period are shown in figure 5. Although it would be desirable to repeat the study of periodicity under a greater range of environmental conditions, the indications are that root growth of loblolly pine does not exhibit a well defined daily periodicity. The slight

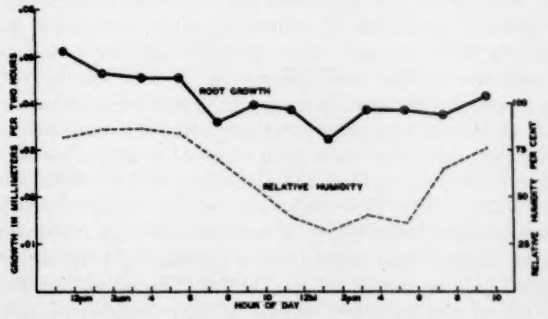

FIG. 5. Root growth in millimeters of loblolly pine seedlings and relative humidity in per cent. for each 2-hour period from 10:00 p.m. June 14 to 10:00 p.m. June 15, 1941. Soil temperature 25° C.

fluctuation in rate appears to have a fair correlation with relative humidity, but there seems to be no evidence of a possible reciprocal correlation with cell division similar to that observed by FRIESNER (9) in seven genera of herbaceous plants.

The average growth rate per two hours decreased slowly from 0.051 mm. at 11:29 p.m. to a minimum of 0.032 mm. at 1:20 p.m. and then increased to 0.042 mm. at 9:37 p.m. The relative humidity was 95% from 9:00 p.m., June 14 to 6:00 a.m., June 15. During the day it reached a minimum of 35% at 1:00 p.m. and then increased to 80% by 9:00 p.m.

These data indicate that although root growth of loblolly pine does not have a marked periodicity, it apparently has some tendency toward a positive correlation with relative humidity and, thus, it is assumed, a negative correlation with transpiration. Although a record of insolation[1] for the period was available no relationship between growth and changes in light intensity during the day was apparent.

Light intensity and root growth

Most studies of the effect of variations in solar energy have compared the dry weights of the roots with the dry weights of the tops. Many have dealt with photoperiodism or effects of duration rather than effects of light intensity. Consequently, such studies are not strictly analogous to those of growth under different light intensities, and they deal with the amount and quality of growth rather than its rate.

Experiments carried out under artificial or natural shading indicate that plants grown under low light intensities produce less roots in proportion to the tops than do those grown in full light and presumably they thus have a slower rate of growth. HAIG (10) and BISWELL (4) reported better root development for plants grown in sunlight than for those in shade. SHIRLEY (24), who studied the effects of shading on several species, found that the ratio of the dry weight of roots to tops increased with increased light intensity. KOZLOWSKI (13) reported a similar response in loblolly pine.

Since photosynthetic rate of loblolly pine has been found to increase quite regularly with increased light intensity (16) one would expect the different amounts of food manufactured to be reflected in root growth. However, there have not been, to the writer's knowledge, any measurements of the rate of root elongation under controlled light intensities. Average midsummer light intensities have been reported to be as low as 267 foot-candles under a red gum–yellow poplar canopy, and for other forest types well above 1000 fc (12). The writer has recorded a light intensity in the unshaded greenhouse below 100 fc on an unusually dark, rainy winter day. Attempts to measure root growth under natural light during the winter months failed because growth ceased. It was thought that insufficient light might be one of the important causes for the cessation of root growth. Consequently, a study was made to determine the rate of root growth at

[1] The record of insolation was furnished by Mr. C. C. Wilson.

different light intensities and the speed and nature of the response to changes in intensity.

Method and Results

Thirteen loblolly pine seedlings about three months old, which had been growing in tubes under natural illumination in the greenhouse, were placed in a dark room for three days to reduce the amount of stored food. The plants were then placed under artificial light in a photosynthesis chamber. (A detailed description of the apparatus used is given by Decker (8).) Twelve 350-watt reflector spot lamps were used as the light source. Light intensity was lowered by interposing layers of cheesecloth and wire screen between the seedlings and the lamps; or, for the lowest intensities, by using fewer lamps. The light intensities tested were: 0, 119, 295, 991, and 5,330 fc. (Measured by a Weston Illumination Meter.)

The temperature of the chamber was held at approximately 25° C throughout the tests. The roots were kept in darkness and at a constant temperature by placing the tubes in a tank of water fitted with a cover so that only the tops of the tubes were exposed to light. The daily period of illumination was 12 hours. Measurements of root growth were made in the morning before the lights were turned on and measurements of light intensity were made immediately afterward.

The average daily root growth for each day of the experiment is presented in figure 6. This graph shows the characteristics of the response to

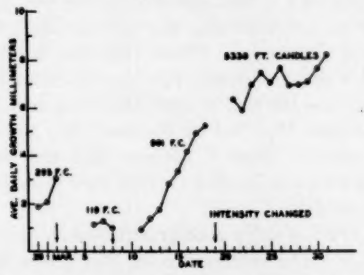

Fig. 6. Characteristics of the average daily root growth in millimeters of loblolly pine seedlings at various light intensities (foot-candles). Soil temperature 25° C.

a change in light intensity. At 295 fc the rate of root growth increased from 1.86 mm. per day for the third day at this light intensity to 3.04 mm. per day for the fifth day. The positive slope of the graph at this intensity indicates that the ultimate daily rate is probably somewhat greater than the rate observed on the fifth day.

The daily growth rate at 119 fc decreased from 1.14 mm. for the fourth day at this intensity to 0.99 mm. for the sixth day. The decrease in the growth rate probably indicates that this intensity is too low for root growth.

A sudden increase in the light intensity from 119 to 991 fc apparently

retarded root growth for approximately three days. SHIRLEY (**24**) has reported a somewhat similar response of loblolly pine to increased light intensity. He also reported an accompanying physical injury very similar to that which might be expected as a result of a water deficit. The average daily rate on the third day at 991 fc was 0.93 mm., which was slightly less than the rate just before the intensity was increased. The average daily rate increased regularly after the third day at 991 fc and was 5.23 mm. for the 10th day. Although two roots had a slower rate of growth on the 10th day after the intensity was increased, the slope of the graph for this period indicates that the ultimate daily growth rate probably would be slightly greater than on the 10th day.

All roots which could be observed and which had not grown during the first two tests commenced growth on the fifth day after the intensity was increased to 991 fc. The formation and growth of lateral roots was especially marked at this intensity, suggesting in addition to more food for root growth the possibility of increased availability of a growth-inducing substance.

No retardation of root growth was observed when the intensity was suddenly increased from 991 to 5,330 fc. The average daily growth rate at 5,330 fc increased gradually from 6.38 mm. at the end of the second day to 8.22 mm. at the end of the 12th day.

Measurements of root growth were made with the seedlings in complete darkness after the completion of the test at 5,330 fc. The average growth rate decreased slightly to 8.11 mm. per day for the first day in darkness and then decreased abruptly to 6.47 mm. per day for the second day and 3.61 mm. per day for the third day. (These data are not included in fig. 6.) Two seedlings had a greater growth rate for the second day than for the first day in darkness and the rate of two others was but slightly less for the second than for the first day; but on the third day the growth rate of all seedlings had decreased. Thus, it appears that the time lag between a change in light intensity and its effect on the root growth of these seedlings was from two to three days.

A graph of the average daily root growth plotted over light intensity is presented in figure 7. The rate shown on this graph is the average rate during the last three days of measurement at each particular light intensity.

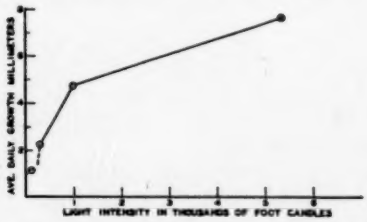

FIG. 7. Relationship between the average daily root growth in millimeters of loblolly pine seedlings and light intensity in foot-candles. Soil temperature 25° C.

This graph is intended to show only the general relationship between root growth and light intensity. The relationship is the same as that found between photosynthesis and light intensity by KRAMER and DECKER (16). There is a rapid rise in the rate of root growth with increased light intensity at low intensities but the increase in growth rate per unit change in light intensity becomes less as the light increases at high intensities.

From this type of response one would expect the growth rate at full light intensity to be only a little greater than the observed rate at 5,330 fc.

Effect of soil temperature on water absorption

The rate of transpiration in unwilted plants is an indication of the rate of absorption of water by the roots, since water must be supplied to the cells to maintain their turgor at about the same rate as it is lost from the leaves (14). Several investigators have observed that water absorption was decreased by cold soil, for example KRAMER (15) reports absorption by loblolly pine to be only 15% as great near freezing as at 25° C.

Because of the direct relationship between root growth and rate of water absorption an experiment was set up to measure transpiration throughout the range of temperatures used in the root growth studies.

Seedlings were grown in 6-inch test tubes so that the weight of the soil would not exceed the capacity of the precision balances to be used. Two tubes containing two seedlings each, and two tubes containing only soil were used for each temperature studied. The seedlings used in these experiments were about four to five weeks old.

Two series of tests were made. The first for soil temperatures of 35° C, 30° C, 25° C, and 20° C and the second series of 20° C, 15° C, 10° C, and 5° C. The tests were made in the greenhouse. The first series was measured from October 18 to October 24, 1946, and the second from October 26 to November 1, 1946. The soil was wetted to field capacity and the tubes were sealed with paraffin. The paraffin was coated with grease, since it was found that paraffin alone did not adequately seal the tubes. No water was added to make up the loss during the period of tests. Consequently, measurements could not be carried on longer than one week, at which time the moisture content of the soil in the tubes with the most rapidly transpiring seedlings had been reduced 15% or to about twice the moisture content at the wilting point.

RESULTS

Measurements were recorded in grams of water lost per day. Before each series was started the seedlings were "standardized" at 20° C and a correction factor was calculated to adjust for differences in seedling size. The weight loss of the tubes with seedlings was also adjusted on the basis of the gain or loss of the tubes without seedlings. Figures 8 and 9 are graphs of the adjusted daily transpiration for each temperature divided by the transpiration at 20° C. The rate of transpiration for the last day of the test was found to increase with increased soil temperature up to 25° C

and then was diminished by increased temperature. In the first series, at high soil temperatures, the transpiration of seedlings growing in soil at 25° C increased in rate from 1.08 [2] on the first day after temperature was raised, to 1.26 on the fourth day. The seedlings growing in soil at 30° C had a relative rate of 1.09 on the first day after the temperature was changed, but from the second to the fifth day the rate differed but slightly from the rate at 20° C. The most pronounced time factor was at the soil temperature of 35° C. The transpiration rate for these plants was 1.14 on the first day and then rapidly and quite regularly decreased to 0.39 on the fifth day (fig. 8). The roots of these plants showed incipient browning near the tips and were not used in series two.

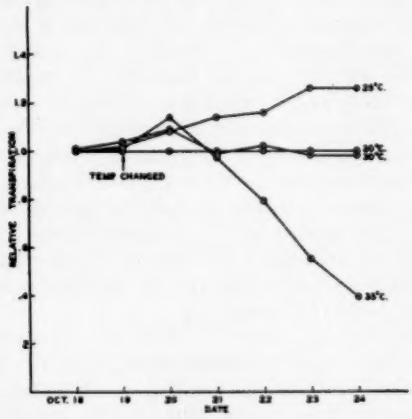

FIG. 8. Relative transpiration rate of loblolly pine seedlings for high soil temperatures. 20° C taken as 1.00.

In series two, at low temperatures, the relative rates of transpiration were always greater at higher temperatures than at lower. A time factor was evident in the relative rates at 15° C, 10° C, and 5° C. The rate of transpiration at these temperatures was less on the second day than on the first. The rate then increased through the fifth day after the soil temperature was lowered. The relative rate at 15° C was 0.86 on the first day and 0.82 on the second day and then increased to 0.93 on the fifth day after the temperature was lowered. The daily transpiration rate at 10° C fell from 0.49 on the first day to 0.39 on the second and then increased to 0.88 on the fifth day after the temperature was lowered. At 5° C the rate decreased from 0.36 on the first day to 0.30 on the second and then increased to 0.68 on the fifth day after the temperature was lowered (fig. 9).

[2] The rate as used here is the relative rate obtained by dividing the transpiration in grams per day for each temperature by the transpiration in grams per day at 20° C for the corresponding day.

Since measurements were discontinued after five days it is unlikely that the final and stable rate was reached for each temperature.

Microscopic examination of the root tips

At the end of the growth measurements for series three and four, two or three roots were selected at random from each temperature. The tips of these roots were removed and fixed in formalin–acetic acid–alcohol solution. They were later embedded in paraffin, sectioned and stained. The potassium iodide test for starch was made on unstained material.

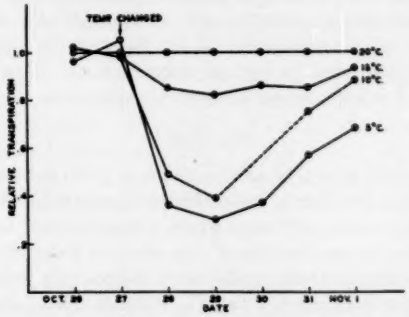

FIG. 9. Relative transpiration rate of loblolly pine seedlings for low soil temperatures. 20° C taken as 1.00. No measurement at 10° C for October 30, 1946.

RESULTS

There was very little difference in cell size or condition between the roots which had been grown at soil temperatures from 5° C to 30° C inclusive. The amount of embryonic tissue and the number of mitotic figures appeared about equal regardless of temperature.

A striking difference from the others was found in the roots of the seedlings grown at a soil temperature of 35° C. These roots had the appearance of dormancy; no mitotic figures were observed and the embryonic region was very small. The region of elongation was short. Thus, there was an abrupt increase in cell size from the embryonic region to fully mature cells. The nuclei of the embryonic cells did not occupy as much of the cell volume and tended to be more spherical than the nuclei of cells of roots grown at lower temperatures. A thick, apparently corky layer of cells enclosed the entire tip. In addition, the parenchymatous tissue between the embryonic region and the root cap was crushed and also appeared corky, forming a second layer of cells covering the embryonic region. During the measurements of root growth it was observed that suberization, as indicated by the browning of roots, apparently proceeded less rapidly at low than at high temperatures; and occurred sooner in sections of the root which extended

across cracks in the soil than in those sections which were in direct contact with the soil. These observations are similar to those of NIGHTINGALE (19) for apple and peach roots. He reported that the relative amount of embryonic tissue decreased as the soil temperature increased from 65° F to 85° F and 90° F, while the amount of external suberized tissue increased. He also reported that roots grown at low temperatures were succulent and those grown at high temperature were not.

The cells of the root caps of roots grown at 5° C contained numerous large starch grains. Starch grains were fewer in number and smaller in size the higher the soil temperature in which the root had grown. Virtually no starch grains were observed in the cells of the root caps of roots grown at 35° C. On the other hand, many of the cells of the cortex of the roots grown at 35° C appeared to contain starch grains. This latter condition was not observed in roots grown at lower temperatures.

DISCUSSION

Low temperature probably acts in many ways to reduce the rate of root growth. Although the rate of water intake is reduced, this is not likely to be limiting except under conditions where a water deficit is produced in the plant. According to observations of CANNON and FREE (6) any decrease in permeability to oxygen would significantly reduce root growth, and if there were a decrease in permeability to CO_2, its slow diffusion out of the roots would probably hinder respiration. Slow absorption of mineral nutrients might conceivably be limiting over a long period of time, but probably not during periods as short as those of this study.

Since food used in synthesis of protoplasm and cell walls must move from the leaves to the roots, low temperature might retard growth by slowing down translocation. CURTIS (7) reported that translocation of carbohydrates through bean petioles is much retarded or stopped at temperatures from 1° C to 6° C but is not greatly affected at higher temperatures up to 25° C. The increased amount of starch in the root tips at 5° C observed in the present study suggests that it was not lack of carbohydrates, but inability to use them that hindered growth at low temperature.

Slower chemical action commonly associated with low temperatures would decrease the respiration rate and thus reduce energy for growth. Furthermore, reduced chemical action would decrease the rate of synthesis of protoplasm. HAWKER (11) has pointed out that production of growth-controlling hormones in some roots is reduced at lower temperatures. Such effects as these may partially account for the slower growth rate at low temperatures. However, growth is such a complex of processes that the effect of low temperature on either physical or chemical processes alone is probably inadequate to account for the slower growth.

High soil temperature, like low temperature, might act in several ways to decrease growth. The marked increase in growth rate followed by a rather regular and slow decrease when the temperature is suddenly raised may possibly be the result of very rapid utilization of food already in the

region of growth. The subsequent slow decrease might be the result of decreasing synchronism of metabolic processes (**21**). BLACKMAN (**5**) has remarked on the similarity in effect of increased temperature on growth rate and rate of enzyme action. The results of the present study suggest that change in growth rate associated with increased temperature is also so complex that it cannot be explained in terms of a single limiting factor. It is more likely that an actual change in protoplasmic structure might occur at supraoptimal temperatures. This is suggested by work of URSPRUNG (**27**). He noted a sudden rise in the amount of water absorbed by living beech and arbor vitae shoots at temperatures between 35° C and 59° C. Any further increase in temperature then caused a decrease in absorption. Dead shoots of these species showed no marked increase or subsequent decrease when tested in the same way. BĚLEHRÁDEK (**3**) noted that several studies had indicated that the viscosity of protoplasm increases as the temperature departs from the optimum. He concluded that the effects of high temperature are too complex to be explained by a single mechanism.

Although low light intensity alone is probably rarely limiting to root growth it may be a contributing factor to the slow growth or the short periods of cessation occasionally found in loblolly pine during the winter.

Summary

A study was made of the effects of soil temperature and light intensity on root elongation of loblolly pine seedlings. The seedlings were grown in a greenhouse in glass tubes filled with soil and maintained at 5° C temperature intervals from 5° C to 35° C. Root growth increased with increasing soil temperatures to a maximum at 25° C then decreased at higher soil temperatures.

The leaf–root ratios decreased with increased temperature above 5° C to a minimum at 20° C but increased at higher soil temperatures.

Suddenly raising the soil temperature from 20° C to 35° C caused a marked increase in growth during the first hour, followed by a decrease, a slight secondary increase, and after 30 hours, almost complete cessation of growth. Growth was not resumed until six days after the soil temperature was lowered from 35° C to 20° C. Part of this delay probably can be attributed to a complete maturation and suberization of the roots which occurred at 35° C.

Except for an increase in starch at the lower temperatures, microscopic examination of the root tips showed only those grown at 35° C to be markedly different from the others. These were rather heavily suberized and had a very small embryonic region. No mitotic figures were observed in these roots.

Apparently the lowest light intensity at which growth took place was between 120 and 295 fc.

Loblolly pine was not observed to have a pronounced daily periodicity in root growth, although there was a decrease in growth rate during the hours when a high transpiration rate could be expected.

A study was made of the transpiration rate of pine seedlings at various soil temperatures. On the fifth day after the temperature was changed from 20° C, the maximum rate of transpiration was at a soil temperature of 25° C. Lower rates of transpiration were observed when the temperature was higher or lower than 25° C. Sudden decrease in soil temperature produced a general lowering of transpiration rate, followed by a gradual increase towards the apparent "stable" rate at the new temperature. Sudden increase in temperature to 35° C caused an acceleration in transpiration rate, followed by a gradual decrease toward the "stable" rate.

The author wishes to express his appreciation to Professor P. J. Kramer, Botany Department, Duke University, for his interest and advice during both the study and the preparation of the manuscript and to Professor C. F. Korstian, School of Forestry, Duke University, for his guidance and suggestions during the study. The author also wishes to acknowledge the cooperation of the North Carolina State Forest Nursery and the Southern Forest Experiment Station, who so kindly furnished the seeds used in this study.

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DYE REDUCTION BY ILLUMINATED CHLOROPLAST FRAGMENTS[1]

A. S. Holt,[2] R. F. Smith and C. S. French[3]

(WITH THREE FIGURES)

Received June 28, 1950

Introduction

It has been demonstrated that when isolated chloroplasts are illuminated in the presence of aqueous solutions of certain oxidants, oxygen evolution occurs (**2, 5, 6, 7, 8, 13**). Provided stoichiometric relationships between the reactants and products prevail, the reaction rate can be measured in terms of oxygen production or in terms of changes in the concentration of whatever other molecular or ionic species are involved, *e.g.*, changes in the concentration of the oxidant, of the reduction product, or of H^+. Of these possibilities, oxygen production, measured by manometric or colorimetric methods, has been most commonly used. Measurements of decrease in the oxidant concentration or increase in the concentration of the corresponding reduction product appear to be limited to determinations with highly colored reagents, *e.g.*, dyes. Hydrogen ion formation or disappearance, which allows the use of acid-base titrations, is limited to special cases, as for example, reduction of an oxidant such as ferricyanide (**7**).

From the foregoing possible methods only two appear to be adaptable to rapid determinations of reaction rates when micro amounts of chloroplast material are to be used. These are: (a) colorimetric determination of oxygen as oxyhemoglobin by the method of Hill (**5**), and (b) determination of the decolorization of oxidation reduction indicators (**8**). Such micro methods require the elimination of secondary influences which can be neglected in manometric determinations. In the determination of oxygen as oxyhemoglobin it is necessary to remove oxygen by preliminary degassing of the reagents and chloroplast material. In the case of reduction of indophenol dyes, the presence of small quantities of natural reducing substances, such as ascorbic acid, introduces a thermal reduction which might invalidate the observations.

In addition to thermal reductions not involving O_2 production, photochemical reduction of certain dyes may occur, which likewise does not involve O_2 production. Such reduction was found when methylene blue or thionine was added to the supernatant fraction from a brei made from

[1] A portion of this work was performed under Contract Number W-7405-Eng-26 for the Atomic Energy Commission.

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leaves of *Phytolacca americana*. On heating this fraction and adding the clear filtrate to these dyes, photochemical reduction was observed again. Such dye reduction is thus a consequence of a photochemical reaction involving substances which are heat-stable and is not to be confused with the reaction responsible for oxygen evolution. Photochemical reduction was again observed if ascorbic acid was added to thionine or methylene blue in place of the filtrate from the heated brei. Hence, the photochemical reduction of thionine by an illuminated spinach chloroplast preparation, reported previously (**8**) is probably not connected with oxygen evolution. Light-stimulated oxidation of ascorbic acid by methylene blue has been reported earlier (**10**).

However, the use of dyes does permit reliable and rapid assays of chloroplast activity if one guards against the possible sources of error pointed out above. Such complications can be avoided by using washed and resuspended chloroplast material; further, their absence can be demonstrated if dye reduction is not effected in darkness by heated or boiled preparations, or by heated preparations when illuminated. If preparations are used in which natural reducing substances are present, manipulations such as dialysis or oxidation with limited amounts of oxidant might be useful for removing their influence. It is with respect to the economy of time and material that dye reduction will find its greatest use, especially when applied to assaying activities of the numerous preparations obtainable by fractionation procedures.

The work to be outlined will include descriptions of the methods, conditions and apparatus used up to the present time for quantitative evaluation of dye reduction. The dyes used included phenol indophenol (red at pH 6.5), 2,6-dichlorobenzenone indophenol, and 2,6-dichlorobenzenone indo-3′-chlorophenol (both blue at pH 6.5). In addition, results of some preliminary attempts to fractionate preparations of disintegrated chloroplasts will be discussed.

Methods

Preparation of chloroplast material

For the fractionation studies, leaves from garden Swiss Chard (*Beta vulgaris* var. cicla) or from market spinach (*Spinacia oleracea*) were used as the source of chloroplasts. The isolation procedure was that previously described (**8**). The intact chloroplasts were suspended in 10% propylene glycol and were fragmented by exposure to supersonic vibrations (**11**) during circulation through a continuous-flow cooling device. The duration of exposure was from eight to 12 minutes. All manipulations used in the isolation of chloroplast material were carried out close to 0° C.

In the early phases of this work chlorophyll concentrations were determined according to the method of French and Anson (**4**). This method was later supplanted by one involving extraction of the pigments with methanol and photometric determination with a Klett colorimeter and the

accompanying red filter No. 66. A calibration curve for this instrument was obtained by transferring the pigment from methanol to ether and determining the concentration of total chlorophyll with a Beckmann spectrophotometer, using the equations of COMAR and ZSCHEILE (**3**).

MEASUREMENT OF THE RATE OF DYE DECOLORIZATION

(a) VISUAL METHOD.—The stock solution used contained phenol indophenol (1.1×10^{-4} M), potassium phosphate (pH 6.5, 0.04 M) and potassium chloride (0.08 M). Chloride was added since WARBURG and LÜTTGENS (**13**) had previously shown that washed or dialyzed chloroplasts are reversibly activated by chloride or bromide. Five ml. of the stock solution were pipetted into a test tube, followed by the sample of chloroplast preparation and distilled water to make 10 ml. Half the contents of the tube were transferred to another test tube containing a small amount of solid reducing agent such as ascorbate or sodium hydrosulphite. The aliquot with reducing agent served as the control from which one judged when the dye in the test sample had been completely reduced. Standard conditions were chosen such that the more active of the preparations to be tested required approximately one minute to reduce the dye completely, the length of time having been shown to be inversely proportional to chloroplast concentration.

(b) PHOTOMETRIC METHOD.—The ideal arrangement for measuring changes of dye concentration would utilize a beam of monochromatic light passing through the reaction mixture at right angles to the beam serving as the source of energy for the photochemical reaction. A single beam was found to be adequate, however. Light from a 1000-watt incandescent lamp was focused on a cuvette ($1 \times 1 \times 4$ cm.) containing dye solution and chloroplast material. The cuvette holder was located directly in front of a window in a metal housing, which contained the photronic cell and color filters. Only the central portion of the beam transmitted through the cuvette passed through the window and fell upon the photronic cell. The metal housing and cuvette were both immersed in a constant-temperature thermostat. The light intensity on the cuvette was varied by means of calibrated screens. Neutral filters were used to reduce the light intensity on the photronic cell. For the dyes mentioned above the following Corning filters were suitable: (a) phenol indophenol: #3387 and #5031; (b) 2,6-dichlorophenol indophenol: #2424; (c) 2,6-dichlorobenzenone indo-3′-chlorophenol: #2404.

Changes in light intensity incident upon the photronic cell were registered by a galvanometer, or more conveniently by a Brown electric recorder (0–10 mv. range; chart speed: 2 div./min., 3 div./inch), which had a response time of 3 seconds for full scale deflection.

For an activity measurement, dye solution and chloroplast material were mixed in the cuvette and placed in darkness in the thermostat for five minutes before being illuminated.

The following conditions required for the method to have quantitative

significance were fulfilled: (a) deflections were proportional to light intensity; (b) a simple relation existed between the logarithm of the deflection and the dye concentration; (c) the initial rate of dye reduction was propor-

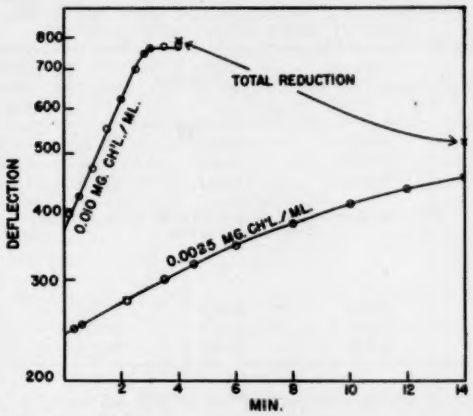

FIG. 1. Reduction of phenol indophenol at different chlorophyll concentrations. 5.5×10^{-5} M dye, pH 6.5, 20° C, 4300 fc, Swiss Chard.

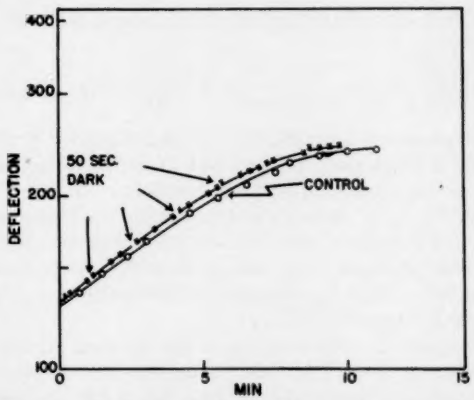

FIG. 2. The effect of interrupted illumination on the course of photochemical reduction of 5.5×10^{-5} M phenol indophenol, pH 6.5, 20° C, 4300 fc, Swiss Chard, 0.01 mg. chlorophyll per ml.

tional to the chloroplast concentration as shown in table I and figure 1; and (d) the rate of reoxidation of the dye was negligible, as shown in figure 2. It is seen that following 50-second dark periods the deflections do not indicate reoxidation. Similar results are obtained with 2,6-dichlorophenol indo-

TABLE I

PHOTOCHEMICAL DYE REDUCTION BY ISOLATED CHLOROPLASTS

DYE: 2,6-DICHLORO-3′-CHLOROBENZENONE INDOPHENOL, 1.18×10^{-5} M, pH 5.9, 0.05 M PHOSPHATE. CHLOROPLASTS FROM *Phytolacca americana*. DEFLECTION D_f, FOR TOTAL REDUCTION FOUND BY ADDING SOLID HYDROSULPHITE.

Mg. chlorophyll per 5 ml.	$\log_{10} \frac{D_{0.5\ min.}}{D_o}$	$\log_{10} \frac{D_{1\ min.}}{D_o}$	$\log_{10} \frac{D_f}{D_o}$	Per cent. reduced	
				0.5 min.	1 min.
0.0016	0.010	0.017	0.180	5.5	9.5
0.0032	0.018	0.032	0.180	10.0	17.8
0.0064	0.036	0.063	0.178	20.2	35.4
Different preparation stored several days at 2° C. 1.66×10^{-5} M dye					
Mg. chlorophyll per 4 ml.					
0.009	0.016	0.029	0.254	6.3	11.4
0.018	0.032	0.060	0.254	12.6	23.6
0.027	0.048	0.090	0.254	18.9	35.4

phenol and the 3′ substituted derivative at pH 6.5 and 5.9, respectively. If, after complete photochemical reduction, one adds a small quantity of an oxidant such as quinone, one finds no indication of any destruction of the dye.

Since two moles of dye are equivalent to one mole of O_2 (**8**) the activity $Q_{O_2}^{ch}$ is given by:

$$\frac{\log D_t/D_o}{\log D_f/D_o} \times \frac{60}{\text{mg. chlorophyll}} \times \frac{\text{moles dye at } t_o}{2 \times t} \times 22.4 \times 10^6$$

in which t is expressed in minutes, D_o is the initial deflection at t_o, D_t is the deflection after a given time interval, and D_f is the deflection after total reduction of the dye when hydrosulphite is added.

Reproducibility is, of course, far better with the photometric method than with the visual method. Occasionally differences up to 15% were found for replicate determinations, using the galvanometer, but in most cases they were less than 10%. With the recorder, determinations are easily obtained with differences less than 3%.

In connection with the determination of the optimum pH for determining the rate of reduction of the 3′ substituted derivative, a decrease in absorption was observed on changing the pH from 6.5 to 5.9 (the optimum pH). This change was from 10.9 to 19.5% transmission when determined with a Beckmann spectrophotometer at 650 mμ. Such behavior is a disadvantage and 2,6-dichlorobenzenone indophenol or any of the dyes listed in table II could well be superior in this respect. The temperature coefficient for the rate of the reaction using the 3′ substituted dye was found to be ca. 1.7 for the temperature range 5–35° C. The dyes listed in table II have not been investigated as to their relative merits with regard to rate of reduction or

TABLE II

INDOPHENOL DYES REDUCED BY ILLUMINATED CHLOROPLASTS

Dye	Color	
	pH 6.5	pH 8.0
Na-2-6-Dibromobenzenone-indo-3′-carboxyphenol	red-violet*	blue*
Na-2-6-Dibromobenzenone-indo-2′-bromophenol	blue*	blue*
Na-2-6-Dibromobenzenone-indo-3′-methoxyphenol	blue*	blue*
Na-2-6-Dibromo-2′-methyl-5′-isopropylindophenol	blue*	blue†
Na Benzenone indo-2′-methyl 5′-isopropylphenol	pink**	blue*
Na-2-6-Dibromobenzenone indophenol	blue*	blue*
Na Benzenone-3′-methyl-6′-isopropylphenol	red**	olive green*

*Decolorization.
**Little decolorization.
†No decolorization.

optimum pH. Preliminary results based on the length of time required for total reduction of equivalent dye concentrations are given for some dyes in a previous communication (8).

Results

Stabilization of chloroplast fragments during storage

Prior to undertaking any fractionation studies two points of interest were the optimum pH for storage of chloroplast fragments and stabilization against activity loss during storage. In figure 3 are presented results ob-

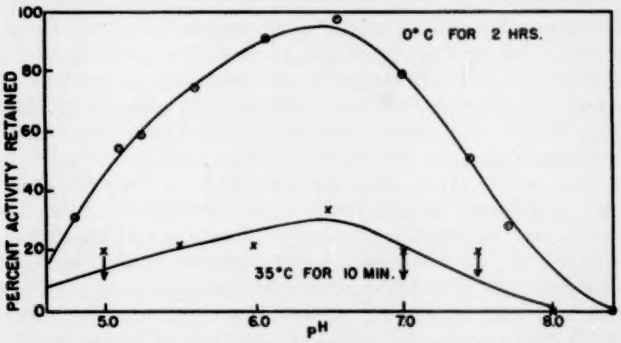

Fig. 3. The loss of activity of spinach chloroplast fragments stored at different pH values.

tained by the visual method comparing retention of activity at two temperatures in 0.02 M sodium sorbitol borate buffer at different pH values. The chloroplast fragments were stored for two hours at 0° C or for 10 minutes at 35° C. The curve for the lower temperature indicates that the activity is retained best in the region between pH 6 and pH 7. Inasmuch

as the visual method could not be used to detect very small differences in activity, the intermediate pH 6.5 was chosen. Results were also obtained by the visual method which led to the subsequent use of propylene glycol as a stabilizer against activity loss during storage at 0° C, pH 6.5. This reagent was diluted with distilled water to various volume per cent. solutions, cooled to 0° C and added to equal aliquots of the fragment preparation. The mixtures were then stored for three hours at 0° C in darkness. Optimum results were obtained with a 10% solution which reduced the activity loss approximately 50% per hour. High retention of activity also appears to be connected with high concentrations of chloroplast material during storage. Other reagents tested included 5% solutions of diethylene glycol, *n*-propyl alcohol, isopropyl alcohol and 10% glycerol, but all of these reduced the activity after storage to a negligible value. Ethanol showed no apparent stabilizing properties when tested over the concentration range 10 to 50%. Strychnine, which has been found to increase the rate of dye reduction (**9**), was ineffective as a stabilizer.

In the case of ethanol, precipitation of the protein such as is observed with intact chloroplasts was absent with disintegrated plastids, and instead a clarification was observed. This was found with concentrations up through 95% ethanol in which precipitation was observed only after standing overnight at room temperature. No reduction of dye was ever observed by the preparations suspended in such concentrated ethanol solutions. Also observed was the shift of the red chlorophyll maximum from 680 to 660 mμ.

Fractionation procedures applied to fragmented chloroplasts

The supernatant obtained from a preparation previously exposed to supersonic irradiation and centrifuged for 10 min. at 7500 × g. (0° C) was precipitated at various pH values. Equal samples at pH 6.5 were diluted to the same volume with McIlvaine buffer so as to adjust to pH values in the range 5.3–6.0. The samples were centrifuged 10 min. (800 × g., 0° C), the supernatant removed, the precipitates resuspended, and the pH readjusted back to 6.5. No marked increases in the activity of any of the fractions so obtained was observed. Total precipitation at pH 4.6 was accompanied by complete loss of activity. Precipitation with neutral salts, *e.g.*, 30 to 40% Na_2SO_4 proved to be no more effective than pH precipitation.

Detergents, sometimes useful in the solubilization of protein without loss of catalytic activity, also failed. Clarification of the extracts was evident with 0.04% Dupanol and 0.12% Wetanol, but no photochemical activity was observed.

Chromatographic adsorption on columns, a technique which has proved to be useful in the isolation of particulate, enzymatically active granules from tumors and mouse melanomas (**12**) did yield active, green material on elution. In pH 5.5 phosphate adsorption was complete in the upper inch of a column of Supercell; elution with neutral buffer or distilled water caused the rapid movement of a light green band away from the major portion of

the adsorbed green material. Neither the strongly adsorbed portions nor the rapidly moving material showed marked enhancement of activity.

Differential centrifugation at different speeds and for varying periods of time at 8000–9000 × g. yielded a fraction which showed an approximately threefold increase of activity. Such increases are minute when compared to the increases of activity shown by many procedures used in enzyme purification. Manometric determinations of the activities of fractions prepared as above confirmed the fact that only small increases were being obtained. The loss of activity during illumination in the absence of chloride, reported previously (1), was observed also with these fractions. This loss occurred regardless of whether the oxidant mixture (ferric oxalate–ferricyanide) was present or not, which suggests that the protective action of the oxidant, pointed out by WARBURG and LÜTTGENS (13), is small in comparison to that afforded by chloride. The activity loss was found to be irreversible in that addition of chloride did not reactivate, in agreement with ARNON and WHATLEY (1).

Discussion

Fractions of the products of chloroplasts disintegrated by means of supersonic vibrations and separated by pH precipitation, salt precipitation, differential adsorption or differential centrifugation did not possess enhanced activity. The assay as conducted was intended to test for an increase in the concentration of some enzymatic component, relative to chlorophyll concentration, responsible under normal conditions for rate limitation of this reaction at "saturating" light intensities (ca. 2500–3000 foot-candles using phenol indophenol as the oxidant). The absence of activity increases might have been due to the need for more complete dispersion and disintegration of the chloroplast components than was accomplished by the procedure described above. This is likely since after 45 minutes of centrifugation at ca. 9000 × g. such preparations yielded only light green supernatant fractions. When the green material in such fractions was precipitated no residual soluble protein could be found.

As pointed out, the lack of precision of the visual method of estimating the point of complete decolorization left much to be desired. The photometric method was developed to detect small changes of activity but this apparatus was not available at the time the above preliminary fractionations were made. However, several-fold increases in activity could not have been overlooked. The photometric method of determining the rate of dye reduction should aid considerably in future studies of a similar nature.

Summary

1. The rate of reduction of dilute solutions of indophenol dyes, approximately $1.0–3.0 \times 10^{-5}$ M, can be used as a quantitative index of the photochemical activity of micro quantities of chloroplast material.

2. The initial rate of dye reduction can be measured within a period of

one minute with a precision of ± 2% using a photometric method and continuous recorder. Less rapid and more scattered results are obtained when the time required for complete decolorization (estimated visually) is used.

3. Reduction of 2,6-dichlorobenzenone indo-3′-chlorophenol is maximum at pH 5.9. The temperature coefficient for reduction of this dye is ca. 1.7 for the temperature range 5 to 35° C.

4. Precipitation, adsorption, and differential centrifugation of preparations of chloroplasts disintegrated by supersonic vibrations failed to yield any fractions possessing noteworthy increases in photochemical activity.

The authors wish to take this opportunity to express their gratitude to the Conway MacMillan Fellowship Committee, University of Minnesota, for financial aid to the senior author and to the Graduate School, University of Minnesota, for a grant in aid. The work dealing with the recording photometric method was done by the senior author while employed at Oak Ridge National Laboratory.

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THE BIOSYNTHESIS OF CARBON-14-LABELED COMPOUNDS. I. THE CHROMATOGRAPHIC SEPARATION OF GLUCOSE AND FRUCTOSE[1]

G. R. Noggle and René A. Bolomey

(with four figures)

Received August 9, 1950

There are a number of compounds of interest to the plant physiologist and biochemist that cannot be synthesized by conventional chemical methods. The plant physiology group in this laboratory has undertaken the isolation of some of these compounds prepared by biosynthetic methods. The present paper describes the general methods used in the biosynthesis procedure as well as the separation of C^{14}-labeled glucose and fructose.

A number of workers have reported on methods for preparing radioglucose and radiofructose. Putman, Hassid, Krotkov, and Barker (**7**) separated radiofructose from radioglucose by recrystallizing the radioglucose in the presence of inactive glucose and fructose carriers. The radiofructose was recovered as a calcium complex which was dissociated with oxalic acid and later crystallized. Their products were cross-contaminated and required additional recrystallization in the presence of hold-back carriers with resultant dilution of the specific activity of the sample. Udenfriend and Gibbs (**10**) prepared radiofructose by the use of paper chromatography, but the method was capable of handling only relatively small amounts of sugar. In order to circumvent the difficulties encountered in recrystallizing sugars in a damp atmosphere, our attention was directed to column chromatography as the method of choice. Column chromatography would have the further advantage that small amounts of the respective sugars could be separated from each other as such without the addition of carriers, thereby maintaining the original specific activity of the sample. The chromatographic procedure based on the work of Lew, Wolfram, and Goepp (**3**) was found to give satisfactory separation and good yields.

Experimental

Photosynthesis apparatus

The glass chamber described by Putman *et al.* (**7**) was employed in preparing small amounts of radiosugars. A second apparatus was constructed for handling large plant samples and is shown in figure 1. The CO_2 generator (B) consisted of a 125-ml. Florence flask. Sufficient $BaC^{14}O_3$ and $BaCO_3$ was weighed into the flask to give a final concentration of 10% CO_2

[1] Work performed under contract No. W-7405-Eng-26 for the Atomic Energy Commission.

in the chamber (A). Water was added to the $BaCO_3$ to form a slurry and the generator was then connected to the plant chamber at (S) with stopcock (T) closed; the two stopcocks (U) and (V) were open. The system was evacuated through (W) to 15 inches Hg. Eighty-five per cent. phosphoric acid was then slowly added to the $BaCO_3$ slurry which was kept in motion with a magnetic stirrer. When the evolution of CO_2 had stopped, the gas generation bulb was gently warmed and air was admitted through (W) until atmospheric pressure was attained. The plant chamber was then placed in the water bath and the lights were turned on. The temperature of the bath and the intensity of illumination determines the relative amounts of monosaccharides and disaccharides formed. During production runs, the C^{14} measuring system (C) was operated only intermittently to check on

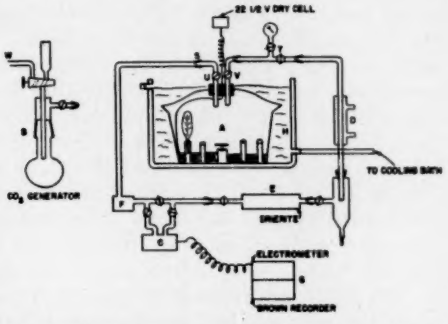

FIG. 1. The photosynthesis apparatus. The plant chamber (A) is a 10-liter Pyrex desiccator. A small fan in the chamber mixes the gas phase. The pump (F) serves to maintain a slow uniform movement of gas through the Geiger-Müller tube (C). The electrometer and recorder (G) attached to the Geiger-Müller tube indicate and record the C^{14} content of the gas phase. The water-cooled condenser (D) and drying-tube (E) dry the gas which is passed through (C).

the disappearance of the C^{14}. At the end of the run, CO_2-free air was swept through the system into CO_2-free alkali. $BaCl_2$ was added to the alkali and the $BaCO_3$ precipitate was collected, weighed and assayed for C^{14}. Generally only small amounts of C^{14} were found in the traps, indicating an almost complete uptake of the CO_2 by the plants.

PREPARATION AND SEPARATION OF RADIOGLUCOSE AND RADIOFRUCTOSE

Detached sugar-beet leaves have been used in most of the experiments to date. Other plant species have been tried and some indicate a very good conversion of CO_2 to sugar. This aspect of the problem will be treated in another paper. The sugar beets were grown in the greenhouse and provided a constant, uniform supply of plant material. The plants were placed in a dark room for 40–48 hours before use. The leaves were then pulled from

the plant and their petioles immersed in water, after which they were placed in the photosynthesis chamber and handled as described under the section headed "Photosynthesis Apparatus."

At the conclusion of the photosynthesis period the leaves were weighed, cut up and extracted with boiling 80% ethanol. The material was slightly cooled and passed through a Waring Blendor. The extract was then filtered through a Buchner funnel containing Filter-Cel and the alcohol removed in vacuo at 35° C. Saturated neutral lead acetate was used to clear the extract and the excess lead removed by H_2S. Deionization of the extract was accomplished by passing it through 60- to 70-mesh Dowex-50 (cation exchanger) and Duolite A-3 (anion exchanger). The Dowex-50 was generally used in the acid cycle (treated with 10% HCl and well washed with water) but it was found that under these conditions some sucrose was inverted to glucose and fructose. About 5 mg. of reducing sugar was recovered after passing 200 mg. of sucrose through the column. This inversion could be prevented by using the column in the sodium form (4% NaOH followed by thorough washing with water). The Duolite A–3 column was treated with dilute NH_4OH (1:2) followed by thorough water washing. The columns (25-ml. bed volume for 9- to 10-gm. leaf samples) were washed with four bed volumes of water. The combined eluate and washings were concentrated in vacuo at 35° C. If separation of the monosaccharides, disaccharides, and trisaccharides was desired, the extract was passed through a charcoal column as described by Noggle and Schumacher (**4**).

If only glucose and fructose were desired, the extract was hydrolyzed with 2 *N* H_2SO_4. The H_2SO_4 was removed by passing through the anion-exchange column and the sugar solution then concentrated to a thick syrup in vacuo at 35° C. The syrup, containing 0.8 to 1.0 gm. of total reducing sugar per 100 gm. of Magnesol, was taken up in a minimal amount of 95% ethanol and added to the Magnesol column. (See following section.) The side walls of the column were rinsed twice with 5-ml. portions of 95% ethanol as soon as all the previous solution was barely imbedded in the column. The sugars were then eluted from the column with 95% ethanol.

Preparation of the columns

After a number of preliminary trials with Magnesol, Florex XXX, Silica, and Alumina (**1**), Magnesol was selected for this work. The Magnesol, a hydrated magnesium acid silicate, was obtained by courtesy of the Westvaco Chemical Division, Food Machinery and Chemical Corporation, South Charleston, West Virginia. Fifty to 100 gm. of the Magnesol were suspended in 95% ethanol. The slurry was poured into a column 3.14 $cm.^2 \times$ 100 cm. long fitted with a glass wool plug. The Magnesol particles were allowed to settle as the excess alcohol filtered through the column.

The free volume of the column as ordinarily measured in a graduated cylinder was not representative of all the columns prepared. It was therefore measured under operating conditions. A weighed amount of Magnesol

was uniformly suspended in a known volume of 95% ethanol contained in a stoppered graduated cylinder and the total volume of the alcohol plus adsorbent was noted. The slurry was then quantitatively transferred to a column of known dimensions and allowed to settle under operating conditions. The bed volume was measured from the dimensions of the column after equilibrium had been reached, and the free volume was calculated. The solid volume of the adsorbent was calculated by subtracting the volume of the alcohol employed to suspend the adsorbent from the total volume measured in the graduated cylinder. The free volume then becomes the difference between the solid volume and the bed volume after equilibrium has been reached under operating conditions.

The preliminary work on evaluating the various adsorbents and eluting solvents was done with mixtures of ordinary glucose and fructose. Ten- to 25-ml. fractions were collected in graduated cylinders as the solution came through the column. A method was needed to follow the elution of the sugars from the column. Since alcohol was the usual eluting solvent, the generally employed copper reducing methods could not be used without first removing alcohol. In the analysis of 50 to 60 separate aliquots this procedure is very time-consuming. However, the well known ring test for sugars with α-naphthol and sulphuric acid may be carried out in the presence of ethanol. This reaction was adapted to the colorimetric determination of sugars in ethanol solutions. Ten milliliters of concentrated sulphuric acid was rapidly added to a mixture of 1 ml. of 1% α-naphthol in ethanol and 1 ml. of an aliquot of the alcohol eluate from the column. The α-naphthol and sugar standards were dissolved in the ethanol concentrations employed for the elution of the sugars from the columns. The solutions and standards prepared in the same manner were allowed to stand for one hour at room temperature and the color intensities then compared in an Evelyn colorimeter equipped with a 540-mμ filter. The intensity and purity of the color was found to change with time. This instability of color renders the method semiquantitative. The method, however, is simple, rapid, and sufficiently accurate to follow the elution of the sugars from the columns and was used in evaluating different adsorbent materials and eluting solvents.

For more precise data on sugar recoveries, the alcohol was removed from an aliquot by heating on a water bath and passing a stream of dry air over the surface. The sample was made up to volume and aliquots analyzed for glucose and fructose. The glucose was analyzed by the method of SOMOGYI (**9**) and the fructose by the periodate method of VAN DER PLANK (**6**), using the Somogyi sugar reagent. The ROE (**8**) method was also used for analyzing the fructose.

When separating sugars labeled with C^{14} it was possible to follow their elution from the Magnesol column by determining the radioactivity of the collected fractions. A 25-lambda (0.025 ml.) sample of 1% detergent (Glim) was placed on a polystyrene film, followed by a 25-lambda aliquot of the

sugar solution. The solution was then dried under a heat lamp and counted with a thin, end-window Geiger-Müller counter.

Paper chromatography was also used to ascertain the location of the sugars in the eluted fractions. Aliquots of the fractions were placed in serial order on Whatman No. 1 filter paper and developed according to the method of PARTRIDGE (**5**). When the paper was sprayed with ammoniacal silver nitrate it was possible to tell at a glance which tube contained only glucose, fructose or both. This method was also valuable for detecting the presence of contaminating sugars. If radiosugars were being separated the location of the sugar on the paper chromatogram could be detected by exposing to Eastman No-screen X-ray film. The sugars could then be eluted from the filter paper and recovered.

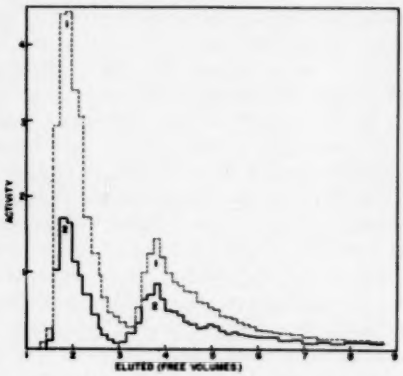

FIG. 2. Elution curve and specific activity of eluted fractions. 1. Radioactivity (counts/sec./ml. $\times 10^{-3}$). 2. Reducing sugar concentration (mg./ml.).

The results in the present work are calculated on the basis of the column free volume (eluted volume). This value is characteristic of the adsorbent, solvent, and column size. All of the experimental values from a large number of column separations are referable to this constant.

Radioglucose and radiofructose were prepared as previously described. The sugars were added to the Magnesol column and eluted with 95% ethanol at a constant rate of about 0.05 ml. per min. per sq. cm. of column cross section (10 ml. per hour). Aliquots of the eluate were analyzed for C^{14} activity and for glucose and fructose. Results plotted against the number of free volumes of eluate are presented in figure 2. The first fraction of the eluate (0 to 1.4 free volumes), containing, as a rule, insignificant quantities of glucose, was discarded. The fraction represented by 1.4 to 2.6 free volumes contained 70 to 80% (depending on the flow rate) of the glucose. No impurities could be detected by paper chromatography of this fraction.

The fraction 2.6 to 3.4 free volumes contained both glucose and fructose.

When this fraction was concentrated and rechromatographed on a fresh column, it was further fractionated into glucose and fructose.

The last fraction (3.4 to 10 free volumes) contained mainly fructose and small amounts of glucose. The amount of glucose usually encountered in this fraction varied between 0.5 and 7% of the total sugar present.

When the flow rate was increased, the degree of cross contamination between the two elution bands was greatly increased. Results presented in figure 3 are typical. Curve 1 represents data obtained at a flow rate of 0.05 ml. per min. per sq. cm. of column cross section. The data from curve 2 were obtained by increasing the flow rate to 0.4 ml. per min. per sq. cm. of column cross section (about 80 ml. per hour) by applying a pressure head to the column.

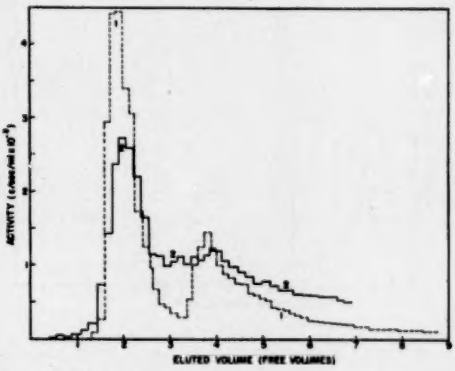

FIG. 3. Effect of flow rate on the shape of the elution curve. 1. – – – .05 ml./min./cm.^{2} 2. —— 0.4 ml./min./cm.^{2}

In general practice, it is better to change the ethanol concentration from 95 to 80% after the greater part of the glucose band has been collected in order to recover the fructose in a smaller volume of solvent. When no interfering sugars are present, this may be done when 2.6 free volumes of the 95% solvent have been collected. When the alcohol concentration is decreased to 80% at 2.6 free volumes, a sharp rise in the fructose concentration will appear at about 3.6 free volumes. Figure 4, curve 1, compares the data obtained in this fashion with that obtained with 95% ethanol as the only developer throughout (curve 2).

The solvent may be made less polar than 95% ethanol with the result that the elution of both sugars will be greatly retarded. This may be of advantage in those cases where a component that adsorbs less easily than glucose is present. Curve 3, figure 4, represents the elution curve of a mixture of glucose and fructose with an eight to two mixture of 95% ethanol and benzene.

When only small amounts of glucose are present in the fructose fraction it is more economical to oxidize the glucose with periodate (**2**) and then deionize the solution with Dowex-50 and Duolite A-3 before crystallizing the fructose.

The alcohol eluate containing the sugars picked up small amounts of impurities as it passed through the Magnesol that interfered with crystallizing the sugars. This contaminant can be removed from the alcohol-free eluate by passing through Dowex-50 and Duolite A–3 ion-exchange resins. The glucose and fructose were then readily crystallized by the methods described by PUTMAN *et al.* (**7**).

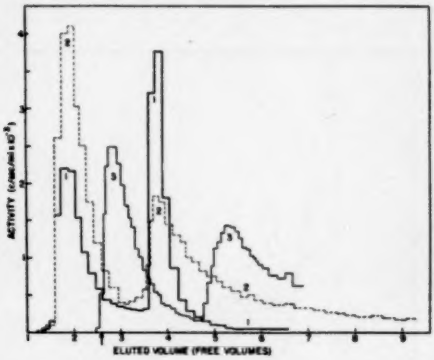

FIG. 4. Effect of solvent on the shape of the elution curve of a glucose-fructose mixture. 1. 95% ethanol up to 2.6 free volumes collected, then 80% ethanol. 2. 95% ethanol throughout. 3. 8:2 mixture of 95% ethanol:benzene.

Summary

1. General methods and apparatus used in the introduction of C^{14} into plants for biosynthesis purposes are described.

2. The sugars were extracted from the plants by boiling 80% ethanol. After removal of the alcohol and precipitation of the proteins, the sugar solution was deionized by passage through cation- and anion-exchange resins.

3. The deionized sugar solution was inverted with acid and the glucose and fructose were separated by passing the solution through a column of Magnesol (a hydrated magnesium acid silicate).

4. The sugar solution was added to the Magnesol column and the column was developed with 95% ethanol. The eluate fraction represented by 1.4 to 2.6 free volumes contained pure glucose. The fraction represented by 2.6 to 3.4 free volumes contained both glucose and fructose. This fraction could be further separated by rechromatographing on a fresh column.

The fraction represented by 3.4 to 10 free volumes contained fructose with only small amounts of glucose. This glucose can be destroyed by periodate oxidation.

5. Pure glucose and fructose can be crystallized from the column eluates.

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BRIEF PAPERS

THE SUGARS OF THE HEMICELLULOSES OF CORNSTALKS[1]

EMMETT BENNETT

(WITH ONE FIGURE)

Received August 5, 1950

Introduction

The qualitative detection and quantitative estimation of individual sugars in a hydrolysis mixture are often attended by some difficulty. The combination of paper chromatography and the refinements in micro-quantitative methods for reducing sugars have been of considerable value in this operation.

The investigation to be reported deals with a study of the sugars from hydrolysates of four hemicellulose fractions obtained from the holocellulose of cornstalks by solvents of increasing strength. The information obtained indicates the presence of two common sugars previously unreported from this source and lends some support to the theory concerning the origin of pentose sugars in hemicelluloses.

Experimental

Hemicellulose fractions indicated as A, B, C, and D in the order of resistance to extraction were hydrolyzed for 10 hours with 4% sulphuric acid in a weight–volume ratio of 1 to 50 in an all-glass assembly. Hydrolysis was brought about in a bath of boiling water. Nitrogen gas was bubbled through the mixture to reduce oxidation to a minimum.

The hydrolyzed sugars were brought into aqueous solution in the usual manner. Positive qualitative tests were obtained for xylose by Bertrand's reaction and by the dibenzylidene dimethyl acetal test (**1**). Arabinose was detected as the osazone and as the diphenylhydrazone. Glucose and galactose could not be detected with certainty by chemical means; mannose was absent. Recourse was then taken to a paper-chromatographic procedure (**2**). The physical system designed consisted of the bowl of an 8-inch desiccator, an 8×18-inch bell jar, a 6×18-inch precipitating jar, a glass plate 2×7 inches, and the cover of a butter dish. The inside bottom of the desiccator and the precipitating jar usually fit together too poorly to afford a level footing for the dish containing the solvent; hence, about 1 inch of sand was placed in the bottom of the desiccator, upon which the precipitation jar could be easily and firmly adjusted. The glass plate was placed across the top of the precipitating jar and bore the dish, which in turn contained the solvent. Paper sheets for the chromatogram were anchored to the bottom

[1] Contribution No. 757 of the Massachusetts Agricultural Experiment Station.

of the dish and draped over either edge as described later. The solvent system used consisted of three parts of ethyl acetate, three parts of water, and one part of glacial acetic acid (**3**). Satisfactory separations could be made in 46 hours at a temperature of about 25° C.

Whatman filter paper #1 in sheets $18\frac{1}{4} \times 22\frac{1}{2}$ were cut into strips $4\frac{1}{2} \times 21$ inches. A fold at right angles to the long dimension was made $2\frac{1}{2}$ inches from one end. This fold should be superimposed upon the edge of the dish containing the solvent. One-half inch below and parallel to the fold, three light pencil dots were placed in a straight line $1\frac{1}{8}$ inches apart and an equal

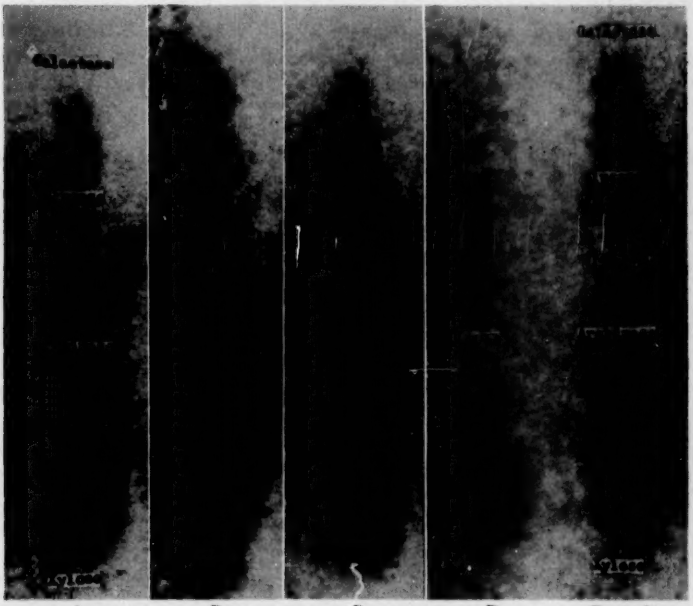

FIG. 1. The combined paper chromatogram of sugars from hemicellulose fractions A, B, C, D compared with a control.

distance from one edge in order to mark the position to be occupied by the test drops. Drops of uniform size are essential and were assured by use of a micropipette. The latter was placed in a fixed position, free from air currents and vibrations. Each drop was equivalent to about 0.007 ml. of water. Stock solutions and unknown solutions are well controlled when the concentration of each sugar is about 1% (**2**).

Control tests were made to determine the order of separation of D-glucose, D-galactose, L-arabinose, and D-xylose. Thereafter each sheet carried three drops; two drops were from an unknown hydrolysate, the third from

a mixture of known sugars which would serve for qualitative and orienting purposes. After the sugars had been separated, their location was determined as described by FLOOD *et al.* (**2**). The results obtained are shown in figure 1. The chromatogram for fraction D indicates only the presence of xylose. Greater concentrations, however, reveal the presence of arabinose, glucose, and galactose, in addition.

A second orienting experiment was conducted relative to quantitative procedures (**2, 4**). It was found that all procedures in this connection could be carried out on a quantitative basis. Data thus obtained were used to determine the molar ratio of the sugars which are shown in table I.

The content of L-arabinose reached a peak in fraction B by the above procedure and by the diphenylhydrazone method. In general, the content of xylose increased as the fractions became more difficult to extract.

TABLE I

THE MOLAR RATIO OF L-ARABINOSE, D-XYLOSE, AND D-GLUCOSE AND D-GALACTOSE IN THE HYDROLYZATES OF HEMICELLULOSE FRACTION.

Fraction	L-arabinose	D-xylose	D-glucose & D-galactose
A	1.0	7.4	1.7
B	2.3	6.3	1.0
C	2.3	9.6	1.0
D	1.0	13.0	1.0

Discussion

It appears significant that the same four sugars are present in all four hemicellulose fractions but in different proportions. A logical treatment of the observation would include first, a statement regarding the origin of the pentose sugars; second, a brief attempt to justify the variation in proportion of the pentoses. If one considers the theory of the origin of pentoses based on the decarboxylation of the uronic acid of the corresponding hexose, it will be evident that qualitatively the proper sugars are present.

If the tendency toward pure xylan in the fraction most difficult to extract is interpreted as being indicative of an aging process which would include qualitative changes and increases in their chain length, one must then account for the elimination of arabinose and the general increase of xylose. The most obvious approach would be to assume that arabinose was never present to any extent in such fractions; furthermore, that the presence of arabinose in different proportions in other hemicellulose fractions represents a stable state. This general statement assumes that there may be distinct hemicelluloses in a given plant. One could also assume that in the type of plant under consideration, arabinose was to some extent a transitory product and disappeared by some such change as occurs in gummosis. The latter process is not fully understood, but it is known that changes in the stereochemical configuration of a sugar residue do occur. The general in-

crease of the content of xylose might be accounted for by this means, and by the decarboxylation process mentioned above.

Summary

Four hemicellulose fractions previously extracted from holocellulose of cornstalks by means of solvents of increasing strength were hydrolyzed. Arabinose, xylose, glucose, and galactose were detected in all fractions. The sugars were determined quantitatively by paper chromatographic procedures. The ratios of the sugars were variable, but in general the content of xylose increased in the fractions most difficult to extract. The presence of the pentose sugars is discussed.

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SUCCINIC ACID CONTENT OF THE COTTON PLANT[1]

DAVID R. ERGLE AND FRANK M. EATON

Received August 11, 1950

Although succinic acid is known to be widely distributed in plant tissues there is only a limited amount of quantitative data on its occurrence. Studies which have been made of the changes in concentration of succinic acid during development of the tobacco plant indicate that it is to be regarded as one of the more active metabolites (**4**).

In a previous study of the organic acids of the cotton plant (**2**) it developed that 14 to 50% of the total organic acids in various tissues were reported, as is customary, as "unidentified acids," *i.e.*, acids other than citric, malic and oxalic. This paper presents data on the concentration of succinic acid in the same or similar tissues.

Materials and methods

Succinic acid was determined on new samples of cotton flowers and seed, otherwise the tissue samples were those used in the previous investigations for the measurement of citric, malic, oxalic, unidentified, and total organic acids.

The succinic acid content of the tissues was determined by the method of PUCHER and VICKERY (**3**), using duplicate 1-gram, oven-dried samples. The determinations of citric, malic, oxalic, and total organic acids were by the methods of PUCHER *et al.* (**5, 6**).

Results and discussion

Succinic acid, table I, on the basis of dry weight, was found to be present in the cotton plant in successively lower concentrations in the leaves, bolls, flowers, seed kernels, petioles, stems, and roots. Although relatively large differences are shown in the concentration of succinic acid between some tissues, the leaves and roots for example, succinic acid was found to comprise only a small part of the dry weight of any of this plant material. Likewise, as shown in table I, its contribution to the total organic acidity of the several tissues approached 10% only in the instances of seed and young bolls; in other tissues it constituted less than 5% of the total organic acids.

The data are of value in that they delimit the extent to which succinic acid can be turned to in efforts at accounting for the organic acids in the unidentified group. The results obtained, table I, show that succinic acid constitutes an appreciable part of the previously unidentified acids occurring in the leaves (17%), 13-day bolls (20%) and mature seed kernels

[1] Published with the approval of the Director of the Texas Agricultural Experiment Station as Technical Paper No. 1325.

TABLE I

SUCCINIC, UNIDENTIFIED, AND TOTAL ORGANIC ACIDS IN VARIOUS TISSUES OF THE COTTON PLANT.

Tissue	Succinic acid, as				Unidentified organic acids, m. e. per 100 g. dry weight	Total organic acids, m. e. per 100 g. dry weight
	Per cent. of dry weight	Milliequivalents per 100 g. dry weight	Per cent. of unidentified acids	Per cent. of total organic acids		
Leaves	0.40	6.8	17.4	2.5	39	276
Petioles	0.13	2.2	5.5	1.4	40	155
Stems	0.09	1.5	5.2	2.1	29	73
Roots	0.07	1.2	3.4	1.7	35	70
Flowers	0.24	4.1	8.4	3.8	49	109
13-day bolls	0.35	5.9	20.3	10.0	29	59
Seed kernels	0.17	2.9	29.0	9.4	10	31

(29%), but only a small part, even though possibly a highly important part, of these acids in the remaining plant parts examined.

In general, the succinic acid content of the cotton plant does not appear to differ greatly, though slightly lower, from that reported for other plants that have been studied (**1, 4, 7**). In tissues of these other plants succinic acid has been found to vary from a trace to 0.8% of the dry weight.

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AUXIN IN RELATION TO LEAF BLADE ABSCISSION

K. Shoji,[1] F. T. Addicott and W. A. Swets[2]

(WITH ONE FIGURE)

Received September 14, 1950

It is a common observation that a leaf stalk usually abscises shortly after the removal of its blade. La Rue (**2**) delayed this abscission of debladed leaf stalks by the application of indoleacetic acid to the stalk. Compounds related to indoleacetic acid also have delayed the premature abscission of leaves and fruits (**1, 3**). These observations suggest that the auxin normally present in an organ is an important factor in delaying its abscission, and that a decrease in this auxin precedes or accompanies abscission. To test these ideas, determinations were made of the auxin content of bean leaves of various ages. This paper presents the results of the auxin determinations and a discussion of their significance.

The first trifoliate leaves of Black Valentine beans, grown in the greenhouse, were used in this investigation. In beans an abscission zone lies between the blade of each leaflet and the leaf stalk. Thus blades differ from stalks in relation to the leaflet abscission zone; tissues of the blade are distal to the zone and tissues of the stalk are proximal to the zone. Samples of blades and stalks were taken from the plants at each age studied, 15, 30, 40, 60, and 70 days after planting. At 15 days the leaves were immature, the blades only partially expanded. At 30 days the leaves were fully expanded. At 70 days the blades were turning yellow and showing other signs of approaching abscission.

Each sample was treated in the following manner: Immediately after cutting it was weighed, put into a container, and frozen at – 15° C. As soon as convenient it was dried in a lyophilizer. The dried material was ground through a 40-mesh screen in a Wiley Mill, and weighed. Each sample was put into a separate 250-ml. Erlenmeyer flask containing 100-ml. of freshly distilled cold ethyl ether, and the flask kept for two hours in a refrigerator at 0° C. The material was then filtered through No. 1 qualitative paper and the residue washed with three 2- to 3-ml. portions of fresh ether. The ether extract thus obtained was evaporated to a few milliliters and with a pipette transferred to a small test tube where it was evaporated to dryness. A measured amount of 1½% agar was added to this dried extract, the mixture shaken vigorously for a few minutes, poured into a $10.8 \times 8 \times 1$ mm. brass mold, and cut into 12 blocks. These blocks were placed on Avena

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coleoptiles, following the standard method described by WENT and THIMANN (5).

The results are summarized in the accompanying figure. The auxin is expressed in indoleacetic acid equivalents (VAN OVERBEEK'S formula) (4). As shown, the immature blades, compared with the corresponding stalks, had a high concentration of auxin. By the 30th day from planting, when the leaves had become fully expanded, their auxin concentration had dropped to a level which stayed constant through the 60th day. By the 70th day when the blades were turning yellow and showing other signs of approaching abscission, their auxin concentration had dropped to the level shown by the stalks. In the stalks a similar but lower level curve of auxin concen-

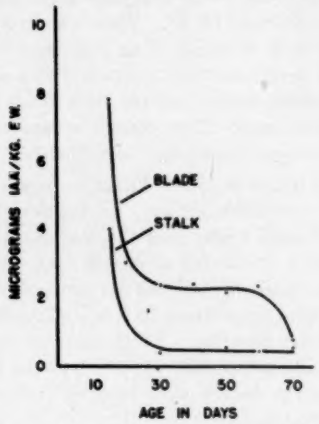

FIG. 1. Changes in the auxin concentration in bean leaf blades and leaf stalks with age. Auxin was determined by the standard Avena assay and is expressed in micrograms of indoleacetic acid per kilogram of fresh weight.

tration was maintained until the 60th day, but did not show a drop at the approach of abscission on the 70th day.

These results show (1) the maintenance of a moderately high auxin concentration in the leaflet blade throughout the period of its normal functioning, a concentration which is approximately three times that in the leaf stalk, and (2) the fall of this auxin concentration to the concentration of auxin in the stalk, occurring as the leaflet yellows and approaches abscission. Thus there is an auxin gradient across the abscission zone while the leaf is active, a gradient which is lost immediately preceding abscission. Experiments with excised abscission zones recently conducted in this laboratory indicate that the auxin gradient, rather than the absolute amount of auxin, controls abscission. These results will be submitted in a later paper.

This paper is based on work sponsored in part by the Biological Department, Chemical Corps, Camp Detrick, Frederick, Maryland, under contract No. W-18-035-CM-208 with the University of California.

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COLORIMETRIC ESTIMATION OF INDOLEACETIC ACID

SOLON A. GORDON AND ROBERT P. WEBER

(WITH ONE FIGURE)

Received October 4, 1950

The wide use of the auxin, indoleacetic acid, in physiological and biochemical experiments has promoted interest in methods for its colorimetric estimation. MITCHELL and BRUNSTETTER (1) have proposed both the nitrite and the ferric chloride–sulphuric acid tests for the quantitative estimation of indoleacetic acid (IAA) in aqueous solutions, basing their suggested procedures upon a study of optimal reaction conditions for these two reagents. According to them, the nitrite method is sensitive to 10 μg. IAA/ml. and develops a red color that is stable after two hours. In several attempts to duplicate their nitrite method using solutions of IAA varying from 20 to 45 μg./ml., we could not obtain a stable red color with IAA at the two hours proposed, or at any other time. A faint pink develops almost immediately which rapidly fades to orange or yellow, depending on IAA concentrations, within $\frac{1}{2}$ hour. If the concentration of nitrite is reduced, the red color becomes sufficiently persistent to be read. Indole likewise gives a strong, relatively stable, red color in this test (cf. table II)—a reaction which is sometimes used as a qualitative test for indole (Nitroso-Indole reaction).

TANG and BONNER (2) have modified the ferric chloride–sulphuric acid method for IAA, combining the iron and sulphuric acid as a single reagent to yield improved sensitivity. However, the color produced is also unstable, rapidly developing and then fading. We have found, as have these workers, that the fading color can be practically dealt with by adopting a standard time between addition of reagent and reading of absorbancy or transmittance.

Both of the methods discussed above possess disadvantages, lacking either specificity, sensitivity, or stability of color complex formed. During a study of the inactivation of IAA in aqueous solutions, it was frequently necessary to assay at one time many samples where the IAA concentrations were low, or where the degree of significance of small differences in concentrations between experimental units required evaluation. Hence, we considered it desirable to re-examine the ferric chloride–sulphuric acid procedure. Several alterations have been made which produce a more stable color, of increased specificity, which changes in density more rapidly with variation in IAA concentration.

1. The procedure of Tang and Bonner can be improved somewhat by reading at 15 minutes after addition of reagent (instead of 30 minutes as they suggest), since the transient color reaches a maximum at the former time. Maximum absorption was found to occur at 530 mμ.

2. Color density and stability as a function of amount of Tang and Bonner reagent added to varying concentrations of IAA were then determined. It was found that a color of maximum density was obtained when the volume ratio of reagent to IAA solution was 1.5 : 1. Moreover, at this ratio, the color becomes stable for several hours 50 to 80 minutes after addition of reagent. With this modification, the $\lambda_{max.}$ is 525 mμ. The increase in sensitivity and stability attained is offset, in part, by increased reactivity of several other indole compounds (table II).

It was considered that a trace of hydrogen peroxide might contribute to an oxidative reaction, hastening the color development, and perhaps increasing its intensity. However, final solution concentrations of hydrogen peroxide from 2×10^{-2} to 2×10^{-6} M resulted in decreased color density and promoted color instability, though the attainment of maximum color was hastened. Lower concentrations of hydrogen peroxide were without appreciable effect.

3. When perchloric acid was substituted for sulphuric acid in the Tang and Bonner reagent, color intensity was improved. By varying the volume

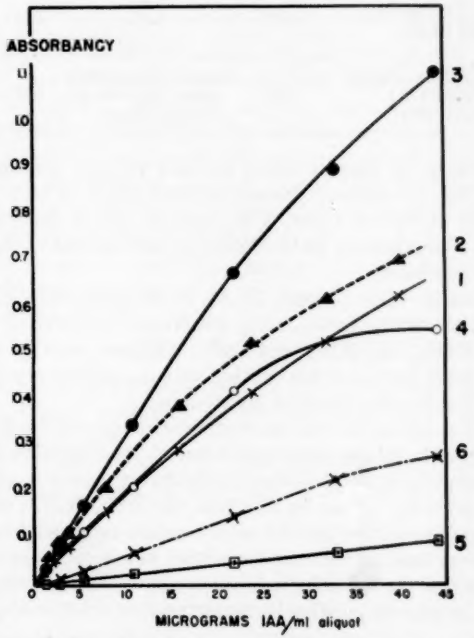

FIG. 1. Concentration-absorbancy curves for indoleacetic acid. Curve numbers correspond to procedures given in table I. Absorbancies determined by Coleman Mod. 14 spectrophotometer, using the shielded cuvette carrier for small volumes.

TABLE I

REAGENTS AND PROCEDURES USED FOR THE COLORIMETRIC ESTIMATION OF INDOLE-3-ACETIC ACID

Reagent	Ml. IAA solution	Ml. reagent	λ_{max} mμ	Remarks
1. Fe-H_2SO_4 1.0 ml. 0.5 m $FeCl_3$ 50 ml. Dist. H_2O 30 ml. H_2SO_4, Sp. Gr. 1.84	1.0	4.0	530	Read at 15′
2. Fe-H_2SO_4 (modified) components same as above	1.0	1.5	525	Read after 75′
3. Fe-$HClO_4$ 1.0 ml. 0.5 M $FeCl_3$ 50 ml. 35% $HClO_4$	1.0	2.0	530	Read after 25′
4. Co-$HClO_4$ 1.0 ml. 0.5 M $Co_2F_4 \cdot 7H_2O$ 50 ml. 35% $HClO_4$	1.0	2.0	528	Boil 10′, cool and read
5. Hg-$HClO_4$ 1.0 ml. 0.5 M $Hg(NO_3)_2$ 50 ml. 35% $HClO_4$	1.0	2.0	555	Read at 15′
6. KNO_2-HNO_3 .05 ml. 2.5% Gum Arabic .025 ml. 0.5% KNO_2 .04 ml. conc. HNO_3	5.0	Reagent components added individually to IAA aliquot	527	Read at 8′ after add'n of HNO_3

and concentration of reagent added to fixed volumes containing varying amounts of IAA, the optimal proportions were found to be 2 parts 0.01 M $FeCl_3$ in 35% $HClO_4$ to 1 part IAA solution. Color density reaches its maximum at approximately 20 to 25 minutes and remains virtually constant for at least three hours.

Other metallic salts (1 part 0.5 M to 50 parts 35% $HClO_4$) were tested with IAA: $SrCl_2$, $TiSO_4$, $SnCl_2$, $Cu(NO_3)_2$, $Ce(HSO_4)_4$, CsCl, $NiCl_2$, $UO_2(NO_3)_2$, $CdCl_2$, $Hg(NO_3)_2$, and CoF_6. Of these, only Co and Hg gave appreciable color reactions, but neither was as satisfactory as the ferric chloride–perchloric acid procedure given above.

In figure 1 are given the concentration-absorbancy curves of those modifications discussed, and the procedures followed are detailed in table I. The relative absorbancy of various other 3-substituted indoles in these methods are given in table II. It can be seen that the $FeCl_3$–$HClO_4$ reagent is not only more sensitive within the IAA concentration range studied, but at the same time shows least interference from other indole compounds. We have, therefore, adopted the following colorimetric procedure for routine estimations of IAA in aqueous solution in concentrations of 0.2 to 45 μg./ml.:

1. To a 1.0-ml. aliquot add 2.0 ml. of $FeCl_3$–$HClO_4$ reagent
2. Read after 25′ at 530 mμ.

   Reagent: 1.0 ml. of 0.5 M $FeCl_3$
   50 ml. 35% $HClO_4$

TABLE II

PER CENT. OF IAA ABSORBANCE DEVELOPED BY VARIOUS INDOLE COMPOUNDS EQUIMOLAR ALIQUOTS (0.125 MILLIMOLAR) TREATED ACCORDING TO THE PROCEDURES OF TABLE I

	1	2	3	4	5	6
	$Fe\text{-}H_2SO_4$	$Fe\text{-}H_2SO_4$ (modified)	$Fe\text{-}HClO_4$	$Co\text{-}HClO_4$	$Hg\text{-}HClO_4$	$KNO_2\text{-}HNO_3$
Indole	12.4	20.6	9.86	16.9	25.0	325.
Skatole	13.4	26.0	10.9	40.8	25.0	24.2
Indole-3-aldehyde .	3.22	3.65	0.86	23.1	8.35	1.96
Indole-3-carboxylic acid	6.20	9.90	4.03	27.0	16.6	10.4
Indole-3-propionic acid	0.74	0.52	0.00	0.96	6.25	8.50
Indole-3-butyric acid	14.4	26.9	11.9	28.9	2.85	11.1
Tryptophan.	0.74	0.42	0.00	1.91	4.17	5.88

Since Beer's law is not followed at high concentrations of IAA, absorbancies obtained are converted to IAA concentrations by a standard curve. We have found that such a curve does not vary appreciably when rechecked with reagent stored in the light at room temperature for over three months. The method has the advantage of being relatively sensitive and is of higher specificity than the methods heretofore used. The stability of the color formed allows a large number of determinations to be made simultaneously without adherence to a fixed time schedule.

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CONSTANT-TEMPERATURE BATHS FOR USE IN FREEZING-POINT MEASUREMENTS

C. M. Johnson, P. R. Stout and G. A. Pearson

Received October 12, 1950

Either salt-ice mixtures or refrigerated thermostatic baths are used for low-temperature baths when making freezing-point-depression measurements of soils, soil solutions, and plant extracts. The use of a mixture of crushed ice and soluble salt such as NaCl or NH_4Cl leaves much to be desired since the lowering of the temperature is difficult to control within the desired limits. If excess NaCl is added to the mixture, the temperature is depressed to such an extent that excessive supercooling results. If only small amounts of NaCl are added, the temperature rises as the ice melts and the NaCl concentration is diminished. Thermostatic baths equipped with refrigerating and heating units are expensive and may not be justified when freezing points are determined only infrequently.

We have used mixtures of sparingly soluble salts and crushed ice to maintain bath temperatures within predetermined limits. Such a cryostat system containing an excess of sparingly soluble salt and finely crushed ice easily maintains a constant temperature within a hundredth of a degree. Different sources of salts, however, may give rise to equilibrium temperatures several hundredths of a degree apart. Temperature control is effected by such a 3-phase system, since as the ice melts more of the salt dissolves, thus keeping the salt concentration constant.

Using handbook data for the solubility of a number of salts at temperatures near 0° C, approximate freezing-point depressions of saturated solutions of these salts were calculated. Salts that seemed likely to give freezing mixtures in the desired range were then selected for testing. An excess of the salt over that required to give a saturated solution when all the ice had melted was added to finely crushed ice and sufficient cold water to yield a slurry that could be conveniently stirred with a mechanical stirrer. Temperatures of the mixtures were measured with a Beckman thermometer. Table I lists the salts that we have found suitable for the preparation of constant temperature freezing baths. Undoubtedly other salts and combinations of salts might be used to extend the range if necessary.

It is helpful to use a Dewar vessel, particularly if the cryostat is to be used over an extended period of time. With this simple apparatus we have had no trouble in maintaining the temperature constant within 0.03° C over a period of several days and to 0.003° C for at least four hours. However, satisfactory results may be obtained in any kind of vessel provided a good excess of salt and finely divided ice is always present and provided the mixture is adequately stirred.

TABLE I

Salt	Grams per 1,000 grams ice*	Bath temperature °C
$Na_2B_4O_7 10H_2O$	20	- 0.40
$Na_2HPO_4 7H_2O$	100	- 0.48
H_3BO_3	30	- 0.70
Na_2SO_4	50	- 1.02
Na_2CO_3	70	- 2.08
$NaHCO_3$	70	- 2.34
$Na_2CO_3 + NaHCO_3$	70 (of each)	- 3.26

* These amounts need not be exact since they are taken only to provide a small amount of solid salt in the mixture.

It has been suggested by BODMAN and DAY (1) in measuring freezing-point depressions of moist soils that the bath temperature be not more than two degrees below the freezing point of the sample. Saturated salt solutions in the presence of ice offer an obvious advantage over refrigerated thermostats in that it is easy to have several baths at different temperatures available at one time so that the freezing-point depressions of samples of widely different colligative properties may be measured at the most suitable temperature.

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NOTES

American Society of Plant Physiologists, Annual Meeting, 1950.—At the annual meetings of the Society during September, 1950, at Columbus, Ohio, the following business was transacted.

J. Fisher Stanfield was reappointed to the Executive Office of Secretary-Treasurer through June 30, 1953. In accord with earlier directives, the Executive Committee appointed five new members to serve as associate editors of PLANT PHYSIOLOGY from July 1, 1951: R. M. Muir (in addition, to act as Secretary of the Editorial Board), through June 30, 1956; C. H. Wadleigh, through June 30, 1955; M. W. Parker, through June 30, 1954; J. B. Biale, through June 30, 1953; Irene H. Stuckey, through June 30, 1952. H. B. Vickery was reappointed to this board, through June 30, 1956. W. E. Loomis was reappointed as Editor-in-Chief of the Monograph Board through June 30, 1953. Further appointments to this board were: F. W. Went, through June 30, 1953 (reappointment); and A. W. Naylor, from July 1, 1951 through June 30, 1954 (to succeed J. W. Shive). R. B. Withrow was reappointed to serve as Representative on the Council of the A.A.A.S. through June 30, 1954. J. Bonner was appointed to serve as Representative to the National Research Council from July 1, 1951 through June 30, 1954 (to succeed B. S. Meyer).

The Memorial Committee, Committee on Chemical Methods, and Committee on Professional Status and Training were voted to be continued active. Two new committees are to be activated through appointment by the President. The first is to consider strengthening coordination of activities between the Physiological Section of the Botanical Society of America and the American Society of Plant Physiologists. It was suggested that this committee review relationships between the two bodies with regard to organization, meetings and publication of abstracts, and general publication problems. The second is to consider and propose a uniform nomenclature on growth substances.

The Executive Secretary-Treasurer indicated that the financial status of the Society was currently satisfactory and should continue to be so, with the increase in dues, voted earlier. He was directed to ascertain the cost of publication of the Constitution and Directory, and if economically feasible, to print the same.

The Editor-in-Chief of PLANT PHYSIOLOGY recommended certain modifications in policy relative to publication of PLANT PHYSIOLOGY. It was voted to authorize the Editor to change printers to effect a saving in costs of publication. He was further empowered to make any future changes in style, format, paper stock, and number of issues per year which in his judgment would effect reduction in printing costs and expedite publication without materially affecting the high quality of our journal.

Dr. Henrik Lundegardh was honored through election to corresponding membership in our Society. Dr. J. W. Shive was honored with the Charles

Reid Barnes Award. Dr. Birgit Vennesland was honored with the Stephen Hales Award.

Extensive discussion concerned the future policy on meeting places, times, and sponsorship. It was voted to meet during September 10–14, 1951 at the University of Minnesota under A.I.B.S. sponsorship. The A.S.H.S. and B.S. of A. will meet then and there, also. It was also voted to meet under A.I.B.S. sponsorship in 1952 and 1953. A.I.B.S. policy includes sponsoring of meetings of relatively smaller size, during the fall, generally at or on a university campus alternating in the East and West, and fostering both symposia and contributed papers. A.A.A.S. policy includes sponsoring of meetings of relatively larger size, during the winter, generally in the larger cities with adequate hotel facilities alternating in the West and East, and fostering symposia as well as contributed papers, with stress on the former.

Papers were of high quality and well presented. We were limited in individual time, by the large number of papers submitted. All sessions were well attended. The program included two symposia, one on Physiological Aspects of Growth Regulators, with Special Reference to Reproduction, the other on Plant Water Relations. Contributed papers covered many phases of physiology; in number, strength in research lay in the fields of Photosynthesis and Growth Regulators with emphasis on biochemical aspects of the phenomena.

The annual dinner was well attended. It proved to be one of the high points, in bringing together those of similar interest for informal discussions and social relations.

The annual business meeting concerned general matters. One phase of discussion pointed up the necessity of encouraging the publication of physiological papers with biochemical aspects, in our journal. Our members are urged to use the pages of our journal whenever possible. Your Society will find ways and means of publication of all papers of high quality in our field.

We are indebted to F. S. Howlett, for over-all arrangements; to C. A. Swanson, in charge of properties; to R. H. Bohning, in charge of the banquet; and many others at Ohio State University and elsewhere (especially the A.I.B.S.) for their cooperative services to our Society.

1949 Science Research Awards of the Oak Ridge Institute of Nuclear Studies.—One of three $500 awards for significant research carried out in the southeastern universities sponsoring the Oak Ridge Institute of Nuclear Studies has been given to Henry C. Harris and his colleagues, Roger W. Bledsoe and C. L. Comar. The award was made for their papers "The Effect on the Growth of Peanuts of Nutrient Deficiencies in the Root and Pegging Zone" which appeared in volume 24 of PLANT PHYSIOLOGY in 1949 and "Absorption of Radioactive Calcium by the Peanut Fruit" which appeared in volume 109 of *Science* in 1949.

Morris A. Raines.—The American Society of Plant Physiologists records with sorrow the passing of one of its members, Morris A. Raines, Professor of Botany at Howard University since 1939. Professor Raines received his B.S. degree from Columbia University in 1915, his M.A. in 1917, and his Ph.D. in botany from the same school in 1922. He was an instructor in botany at West Virginia University from 1920 until 1922. Professor Raines had been a member of the A.S.P.P. continuously since 1931. He died on July 1, 1950, at the age of 56.

Advances in Agronomy, Volume II.—A. G. Norman, Editor. Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 407 pages, 1950. $7.50.

This volume continues the critical review of recent developments in subjects of interest to agronomists. The important aspects of cotton production are discussed by nine specialists and coordinated by J. E. Adams. The other topics reviewed are: Soil nitrogen by L. E. Ensminger and R. W. Pearson; Vegetable production by J. E. Knott and O. A. Lorenz; Prairie soils of the Upper Mississippi Valley by G. D. Smith, W. H. Allaway, and F. F. Riecken; Ladino clover by G. H. Ahlgren and R. F. Fuelleman; The control of soil water by E. C. Childs and N. Collis-George; Preservation and storage of forage crops by R. B. Musgrave and W. K. Kennedy; The reclamation of coal mine spoils by H. Kohnke; Irrigated pastures by W. Keller and M. L. Peterson. The table of contents and organization of each review along with complete author and subject indices contribute considerably to the value of the volume as a reference work.

Plant Biochemistry.—James Bonner. Academic Press, Inc., 125 East 23rd Street, New York 10, New York. 537 pages, 1950. $6.80.

Plant scientists generally will welcome this publication on the biochemistry of higher plants. Timely in its approach that certain metabolic pathways are common to many kinds of organisms, the integration of the evidence for such pathways in higher plants represents a valuable service. The book is not merely a collection of facts but a comprehensive account of metabolism in the plant. It describes those processes peculiar to plants and general biochemistry as it applies to plants indicating those phases which require investigation in view of knowledge of microorganisms and higher animals. The book will serve equally well as a textbook for students of plant biochemistry and as a reference volume for students of plant physiology if they have some training in organic chemistry. The subject matter is organized under four headings: Carbohydrates and carbohydrate metabolism; The cell wall and cell wall metabolism; Plant acids and respiration; Metabolism of nitrogenous compounds; Secondary plant products; and Certain aspects of plant growth, which includes a discussion of the plant growth

substances and photosynthesis. Each chapter includes a list of general references and a list of research papers. Author and subject indices complete the book.

Problems of Morphogenesis in Ciliates.—ANDRE LWOFF. John Wiley and Sons, Inc., New York, New York. 103 pages, 1950. $2.50.

In this short but thought-provoking series of essays, the author has presented lucidly some of the basic problems of development and evolution in a group of relatively highly differentiated, unicellular, ciliated organisms. He presents and discusses alternative theories to explain some of these problems. Careful, well illustrated, descriptions of the position, and differentiation of several of the cytoplasmic organelles of the ciliated, monoenergid cells are given. Cilia-producing organelles such as blepharoplasts (kinetosomes) and kinetodesma are discussed in detail in terms of their relation to plasmagenes and other cytoplasmic factors believed to control development and differentiation. Students of cytoplasmic particles, their relation to the chemistry of metabolism and to reproduction, genetics and evolution will be stimulated by Dr. Lwoff's direct, lively style.

Cell Physiology and Pharmacology.—J. F. DANIELLI. Elsevier Publishing Company, Inc., 250 Fourth Avenue, New York 3, New York. 156 pages, 1950. $3.00.

Published lectures are often noteworthy for their direct and concise treatment of subject matter. This is particularly true of this volume based upon lectures given at University College, London. Unencumbered with exhaustive citations of the literature and divergent opinions the book is an interesting and readable account of drug action based upon our knowledge of cell physiology. The limitations of this knowledge and the need for further biological research on drug action are stated with a precision that reflects the author's experience in the search for new drugs. The topics include: The cell as a physico-chemical unit; Possible actions of drugs on surfaces; Membrane permeability and drug action; Enzymes and drug action; The actions of narcotics; Responses of cells on the biological level. The contribution will be of considerable general interest to physiologists as well as pharmacologists, chemists and medical men.

Carotenoids.—P. KARRER and E. JUCKER. Translated and revised by E. A. Braude. Elsevier Publishing Company, Inc., 250 Fifth Avenue, New York 1, New York. 384 pages, 1950. $8.50.

The extensive literature appearing in the last 15 years on the carotenoid pigments and the increasing importance attached to them in physiology led to the original monograph by Karrer and Jucker published in 1948. The new edition in English includes corrections and material added by Braude. The clear presentation and excellent organization contribute much to the value of the book. One part of the volume considers the mode of occur-

rence, detection and estimation, formation and physiological significance, isolation, chemical constitution and methods of elucidation, synthesis, and distribution of carotenoids in nature. A second part describes the carotenoids of known constitution in detail and also those of partly or completely unknown structure. References to the original literature, color plates showing the crystalline form of some carotenoids, light absorption curves, an index of vegetable and animal sources of carotenoids and a subject index complete the volume.

An Introduction to the Embryology of Angiosperms.—P. MAHESHWARI. McGraw-Hill Book Company, Inc., 330 West 42nd Street, New York 18, New York. 453 pages, 1950. $6.00.

Dr. Maheshwari has contributed greatly to progress in the study of embryology in plants by bringing together in one book much of the mass of information that has accumulated over the years on the embryology of flowering plants. In this collation of material from hundreds of papers he has included in his broad definition of the term embryology, microsporogenesis, megasporogenesis, the development of male and female gametophytes, the events leading up to and including fertilization, in addition to the development of the embryo and endosperm. Plant physiologists will be interested in the summary section on experimental embryology. The ample bibliographies listed at the ends of the several chapters add to the value of the book as a reference volume. Most of the 216 illustrations are taken from original articles and are well selected to clarify the developmental aspects of the subject. The style is essentially that of a literature review but it is augmented by the author's ability to integrate.

Colorimetric Determination of Traces of Metals.—E. B. SANDELL. Second Edition. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 673 pages, 1950. $9.00.

This is a revised and enlarged edition of the book which was published in 1944 as Volume III of a series on Chemical Analysis. The new edition contains further applications of previously known colorimetric reagents with some elimination of older procedures. The plan of the book is the same as before with a general section on colorimetric trace analysis, including methods, limitations, precautions, procedures for the separation and isolation of traces of metals, colorimetry and spectrophotometry in trace analysis, and general colorimetric reagents. The special part gives detailed procedures for the determination of traces of 45 metallic elements and the rare earths. Author and subject indices are included.

Apples and Apple Products.—R. M. SMOCK and A. M. NEUBERT. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 486 pages, 1950. $7.80.

The second volume in this series on economic crops deals with the apple

fruit after harvest. The production of the fruit is considered only as it may affect the fruit following harvest. Information on the handling, storage and processing of the apple is arranged systematically and stated concisely. Citations of some 900 papers are included for the benefit of specialists in any phase of the production and utilization of crops. Other sections consider the distribution, varieties, development and structure, and chemistry and physiology of the apple. Detailed descriptions are given for the processing of the many products derived from this fruit. An appendix with apple crop statistics for recent years is included with author and subject indices. The book is a valuable addition to the economic crops series presenting critical discussions on the problems of production specialists, food processors and nutritionists.

Iodine and Plant Life. Annotated Bibliography 1813–1949.—Chilean Iodine Educational Bureau, Stone House, Bishopsgate, London, EC2. 114 pages, 1950.

In the last 25 years a large number of papers have appeared which deal with the physiology and biochemistry of iodine in plants. Therefore the compilers of the Chilean Iodine Educational Bureau have prepared this bibliography excluding papers on iodine chemistry of soils, iodine content from the standpoint of human and animal nutrition, and the uses of iodine in medicine. The bibliographies of these topics are to appear later. In the present volume 749 papers are listed in separate sections on marine plants, land plants, micro-organisms and plant protection. A valuable review of the literature of iodine and plant life, an author index, a subject index and a key to abbreviated titles of periodicals complete the volume.

Morphology and Taxonomy of Fungi.—Ernst A. Bessey. Blakiston Company, Philadelphia, Pennsylvania. xiii + 791 pages, 1950. $7.00.

As the title indicates, this book is concerned with morphology and taxonomy, such mention as is made of physiology and genetics being avowedly incidental. The aim of the book is to bring together the essential characteristics of the various groups of fungi as understood today. In this it is eminently successful. The extensive bibliographies, the numerous excellent illustrations and the guide to the literature for determination of fungi serve to make this a standard and authoritative reference book as well as an outstanding text. The treatment of the Fungi Imperfecti is much more extensive than in other texts covering the same ground. In view of the very great importance of these forms in physiological work at the present time, this is a most valuable feature.

Diseases of Cereals and Grasses in North America (Fungi, except Smuts and Rusts).—Roderick Sprague. The Ronald Press Company, 15 East 26th Street, New York 10, New York. xvi + 538 pages, 1950. $7.00.

This is a compilation of what is known about the fungi which cause or are suspected of causing diseases of members of the grass family in North

America, including Central America, and Hawaii, but excluding the West Indies. The fungi are arranged alphabetically within the major groups: Phycomycetes, Ascomycetes and the form orders of the Fungi Imperfecti, the last occupying about two-thirds of the text. The rusts and smuts are omitted, presumably because there is adequate information about them available elsewhere. Each group is preceded by a key to the genera included in the text. There is a short glossary and an extensive bibliography. Wherever it is known, reference is made to the influence of environmental factors on host and parasite with reference to incidence of disease. The illustrations are for the most part clear and helpful.

Erratum.—The editor's attention has been called to an error in the paper, "Soil aeration as a factor in water absorption by the roots of transpiring plants," by Robert M. Hagan, which appeared in the October 1950 number of PLANT PHYSIOLOGY. Table I which is referred to in the text on pages 752 and 755 was omitted. The table, as it should have appeared in this paper, is as follows:

TABLE I

SUMMARY OF RECOVERY RATIOS* OBTAINED IN TIME OF RECOVERY EXPERIMENTS

Number of runs	Period of gas saturation prior to irrigation (min.)	Recovery ratios	
		Determined ratios	Mean ratio
		Saturation with nitrogen	
5	10	0.90, 2.0, 1.3, 1.5, 1.0	1.3
1	20	2.2	2.2
3	40	1.3, 1.3, 1.1	1.2
3	60	1.4, 1.6, 1.1	1.4
3	120	2.0, 1.4, 0.93	1.4
1	360	1.3	1.3
3	720	2.0, 1.2, 1.6	1.6
		Mean nitrogen recovery ratio = 1.4 ± 0.085**	
		Saturation with carbon dioxide	
7	10	2.5, 2.3, 2.6, 3.0, 2.9, 2.3, 4.2	2.8
1	40	1.8	1.8
1	60	2.1	2.1
3	120	2.6, 2.9, 2.5	2.7
		Mean carbon dioxide recovery ratio = 2.6 ± 0.17	

*The recovery ratio is defined as equal to the recovery time of treated plant divided by that for the air check plant.

**Error expressed is the standard error of the mean.

PREPARATION OF MANUSCRIPTS FOR PUBLICATION IN PLANT PHYSIOLOGY

The following suggestions are made to authors writing papers for this journal in order to minimize revisions and editorial corrections. Though many points may appear arbitrary or trivial, attention to details is necessary to secure uniformity of format and style. The membership dues and subscription fees go almost entirely to publication costs, as all of our editors give their service without financial recompense. Hence the cooperation of authors in preparation of manuscripts minimizes expense and permits greater liberality as to length and number of papers published. Authors will find the following concise publications useful in preparation of manuscript copy: A. J. Riker, The Preparation of Manuscripts for Phytopathology. Phytopathology XXXVI, pages 953–977, 1946; and S. F. Trelease, The Scientific Paper: How to Prepare It. Williams and Wilkins Company, Baltimore, Maryland; 1947.

1. **Length.** The allowable length of papers is 20 printed pages, with tables and figures not to exceed 25 per cent. of the total. Authors will be billed at cost for overrun and excess of figures and tables.

2. **Format.** In the preparation of typescripts, authors are requested to examine a printed copy of the journal of PLANT PHYSIOLOGY to note the position of headings, general organization, punctuation, abbreviations, and the methods of citing literature particularly, in order to bring the script into conformity with the general style of the journal. Leave a 3-inch margin at the top of the first page, liberal side margins, and double spacing of lines to permit proofmarking and required editorial changes.

3. **Headings.** Leave all headings unscored and scientific names without underlining, as the editors will proofmark these items in accordance with the practice of the journal. Generic names used alone are usually capitalized but not italicized. In general, do not underscore any words.

4. **Footnotes.** Avoid footnotes as far as possible by placing such items under acknowledgments or at end of summary. When footnotes are used, they should be numbered with Arabic numerals.

5. **Figures.** Copy for figures (photographs and drawings) should not exceed $8\frac{1}{2} \times 11$ inches. Retain original drawings as a safeguard against loss. If the figures submitted are larger than $8\frac{1}{2} \times 11$, they will be photographed at this office and the author will be billed for the same. Author's name and figure number must be written on the back of copy for each figure. Do *not* put legends into or on the figure, either front or back. Type all legends *double* spaced, properly numbered to correspond to the figures, on a *separate* sheet of your manuscript. Graphs and drawings should be inked with fairly heavy lines and with lettering of approximately uniform size to insure clarity of printed figures after reduction. Photographic prints should be submitted on gloss-finish paper. Limit copy to desired visual details, trim off irrelevant foreground and minimize blank space, as charges for cuts are based on area. Unless otherwise directed, the editor will proofmark copy to determine actual size of printed figures. Old cuts will be sent to authors after printing for cost of postage if printer is so directed when galley proof is submitted.

6. **Tables.** Place each table on a separate page; do not crowd too much material into a single table. Note size of printed page (width and length, $4\frac{1}{2} \times 7\frac{1}{2}$ inches) and gauge tables accordingly, leaving ample space in margins and in the interior for editorial marks. If at all possible, arrange tabular data horizontally on the page in order to obviate tilting of printed table. Use horizontal lines (rules) at top and bottom, but omit or employ such rules sparingly in interior of tables. Use vertical rules between columns. Rules and spaces (leads) are usually used in the final printed tables to separate sets of data. For footnotes to tables use the asterisk, dagger, double dagger, and section in this sequence as needed. Tabular material should be used sparingly because of its high cost.

7. **Acknowledgments.** Place acknowledgments at the end of the paper just below summary.

8. **Address.** The name of your institution and its address should also be given at the end of the summary.

9. **Literature Cited.** Use separate pages for the literature cited and type *double spaced.* Give complete citations, author, title, journal name, volume number, inclusive pages, and year. (See any literature list in a recent issue of the journal for examples.) Punctuate according to the examples, and leave no citations incomplete. Write out names of states in full. Note proper abbreviations of journals. An error frequently noted in manuscripts with long bibliographies is failure to mention in the text all citations listed. Authors are requested to check all citations in the bibliography by careful proofreading of manuscripts prior to submission.

10. **Formulae.** Use mathematical formulae only when necessary, and be conservative in the use of space.

11. **Galley Proof.** Correct all errors clearly and return galley promptly to editor. Authors will be billed at cost for alterations in the galley proofs, other than corrections of printer's errors, involving excessive changes from manuscript copy.

12. **Mailing.** Send original copy of manuscript to editor, W. F. Loehwing, University of Iowa, Iowa City, Iowa. Retain a carbon copy to insure against loss, and to consult in case of need. Prompt return of revised manuscript copy and galley proof to editor greatly expedites publication. Write across top of title page of manuscript directions for mailing of proof, thus: Mail proof to (author's name and address). If authors wish to have original manuscripts or copy for illustration returned subsequent to publication, the editor should be so informed at the time proof is returned.

13. **Reprint Order.** These should be sent to the editor with corrected galley proof. Late orders for reprints entail extra work and expense by printer for which charges must be made to author. Type is destroyed immediately after printing and hence it becomes impossible to supply reprints after publication of papers. Send Reprint Order with *corrected galley* even if formal requisition or purchase orders for institutional agencies follow later.

14. **Abstracts.** Authors are requested to submit typed abstract on form provided when corrected galley is returned to editor who will insert proper volume and paging for use by Biological Abstracts. If authors care to type a carbon copy of abstract on plain paper, the second abstract will be sent to Chemical Abstracts or some other suitable abstract journal.

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ings and photographs should not exceed 8.5 × 11 inches in
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the text presented in concise form. Instructions to authors
of manuscripts are given in the January number, Volume
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